Fetal programming in pregnancy-associated disorders
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In utero sFlt-1 exposure differentially affects gene expression patterns in fetal liver

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Under revision
Abstract

The soluble fms-like tyrosine kinase factor 1 (sFlt-1) has been identified as a major contributor to angiogenic dysbalance during preeclampsia. Several studies have shown that preeclampsia can lead to metabolic disturbances in offspring. However, little is known about the effects of sFlt-1 on the fetal health. In this study, we aim to evaluate the effects of high sFlt-1 concentrations during pregnancy on the fetal liver physiology in a rat model.

Sprague-Dawley dams were exposed to control or sFlt-1 adenovirus during the mid-gestational period, and later they were subclustered according to concentrations (low and high sFlt-1 based on the sFlt-1 plasma levels at term of pregnancy), comprising three experimental groups. Fetuses exposed to high sFlt-1 concentrations in utero showed fetal growth restriction and brain-sparing effects in comparison with the low sFlt-1 and the control group of fetuses. In continuation, the microarray analysis of the fetal liver of the high sFlt-1 group showed significant enrichment of key genes for the fatty acids metabolism and Ppara targets. In addition, using pyrosequencing, we found that the increased Ppara expression in the high sFlt-1 group is accompanied with decreased methylation of its promoter.

Our data show that high sFlt-1 concentrations during pregnancy have detrimental effects on the fetal growth and liver physiology, including upregulation of the fatty acid metabolism genes and the Ppara targets in the fetal liver. Changes in DNA methylation point to potential long-term effects for the offspring.
Introduction

The fetal programming hypothesis proposes that the adverse intrauterine and early life environment can shape the individual towards susceptibility to cardiometabolic diseases [1]. This concept is supported by many studies that have shown that malnutrition, toxins, smoking, and maternal diseases during pregnancy affect the birth weight of the offspring, resulting in an aberrant response of the cardiovascular and metabolic system in later life[2–5]. Furthermore, the opposite situation of increased birth weight or macrosomia also contributes to metabolic programming and later-life morbidity, primarily via altered body composition at birth [6–8].

Preeclampsia is a pregnancy-associated disorder, usually complicated with fetal growth restriction and affects 3-7% [9] of the population worldwide. It is diagnosed with hypertension and proteinuria and poses a major threat to the maternal and fetal health[10]. As a multifactorial disease, it is associated with several pathophysiological pathways including angiogenic imbalance with a predominance of soluble fms like tyrosine kinase-1 (sFlt-1), which is 2 to 5 fold increased in preeclamptic patients [9,11]. sFlt-1 serves as an antagonist of placental growth factor (PlGF) and vascular endothelial growth factor (VEGF), thus promoting hypovascularization, endothelial dysfunction and vasoconstriction in the mother with concomitant hypoxia [12].

Recent studies suggest that sFlt-1 may play a role in lipid and protein metabolism. The circulating plasma proteome of the dams exposed to sFlt-1 shows increased apolipoprotein fractions that are mediators of cholesterol esterification and removal of excess cholesterol from tissues[13]. Moreover, the fatty acid availability is increased in sFlt-1 induced preeclampsia due to increased expression of fatty acid translocase in sFlt-1 overexpressing placental tissue[14]. Additionally, it was reported that liver X receptor and retinoid X receptor (LXR/RXR) pathway is upregulated in sFlt-1 induced preeclampsia which is a substantial pathway in the regulation of lipid metabolism, and inflammation [13].

Although increased concentrations of sFlt-1 contribute to the pathogenesis of preeclampsia and fetal growth restriction [15], the specific effects of sFlt-1 on the developing fetus are not well understood. With this study, we aimed to evaluate (1) the effects of sFlt-1 on the fetal development, (2) adjustments of the fetal liver gene expression, and (3) whether prominent changes in gene expression are accompanied by prospective changes in the fetal liver DNA methylation.
Materials and methods

Animals

All animals were housed and handled according to the institutional guidelines following Dutch legislation. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Groningen (DEC-RUG 6420E). Female Sprague-Dawley (SD) rats (Charles River, France) were housed in a light and temperature controlled facility (lights on from 7:00 am until 7:00 pm, 21°C). Food and water were provided ad libitum. Vaginal smears were taken daily and when the females were in a pro-oestrus phase of their cycle, were housed with a male overnight. The following day, if spermatozoa were present in the vaginal smear, was considered as day 0 of gestation. At day 8 of gestation, animals were randomly divided into two groups, control (n=5) and sFlt-1 (n=7), receiving either control Ad-null or adenovirus overexpressing sFlt-1 (Ad-sFlt-1), respectively. We distinguished either ultra-high or very low sFlt-1 concentrations in our sFlt-1 group, so we used a cutoff point of 1000 ng/ml sFlt-1 in plasma to continue working with high (n=3) and low sFlt-1 (n=4) group of dams.

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, the Netherlands) and Ad-sFlt-1 (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). The clear thick adenoviral band was transferred to a DG column (Biorad, Temse, Belgium) for washing and elution. 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). 1x10^12 PFU of adenovirus expressing an empty vector or mouse sFlt-1 were injected via the tail vein on gestational day 8.

Blood pressure measurements

On gestational day 19, the systemic blood pressure for all groups was assessed under anesthesia (100% O2, 0.8mL/min, 5% isoflurane for induction followed by 2%) with the aortal catheter positioned in the abdominal aorta and connected with a bed-side monitor (Datex-Ohmeda, Cardiocap/5). Blood pressure measurement values were recorded 30 seconds after cannulation.
**Analysis of blood and urine samples**

On gestational day 12, maternal blood was collected in EDTA containing tubes via the tail vein. Within half an hour the blood was centrifuged for 20 minutes at 1000 rpm and the plasma was stored at -80°C until analysis. On gestational day 19, animals were anesthetized with isoflurane inhalation and the abdominal cavity was opened. After blood pressure measurements, blood was collected via the abdominal aorta and centrifuged as stated above to collect plasma. Plasma was stored as stated above. 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 heparin-coated capillary tubes (Vitrex Medical A/S, Herlev, Denmark). SFlt-1 concentrations in plasma were determined using a mouse sFlt-1 ELISA kit (R&D Systems, Inc., Minneapolis, MN, USA). 24 urine samples were collected by placing the pregnant dams in metabolic cages at gestational day 16 and 17, and the protein levels were determined using Pierce method. The concentration of proteins (mg) per sample was multiplied by the 24-hour urine volume.

**RNA isolation**

Total RNA was isolated from fetal livers collected on gestational day 19 with allprep DNA/RNA mini kit (Qiagen, Venlo, the Netherlands), following the manufacturer’s protocol. RNA quality and quantity were assessed with Nanodrop 2000c (Thermo Scientific, Pittsburgh, PA, USA). RNA was immediately stored at -80°C until further analysis. RNA integrity was further verified on an Agilent 2100 Bioanalyzer, using the Eukaryote Total RNA Nano Assay according to manufacturer’s protocol (Agilent Technologies, Amsterdam, the Netherlands). Samples that had an RNA integrity number (RIN) above 9.0 and no RNA degradation products were considered acceptable for microarray hybridization.

**Microarray hybridization and analysis**

Total RNA isolated from fetal livers (5 samples per group: control and high sFlt-1) was used for microarray analysis. Labeled cDNA was prepared from 100 ng RNA using the Ambion Whole Transcript Expression Kit (Life Technologies, Carlsbad, CA, USA) and Affymetrix GeneChip WT Terminal Labeling Kit (Affymetrix, Santa Clara, CA, USA). All samples were hybridized to Affymetrix GeneChip Mouse Gene 1.1 ST arrays according to standard Affymetrix protocols. Array images and normalization was performed with Bioconductor software packages (Lin 2011). Probes sets were assigned to unique gene identifiers (IDs) of the Entrez Gene database, resulting in 19,357 assigned Entrez IDs.
Bioinformatics analysis

The microarray covered 19,357 genes, and only the ones with an intensity value of >20 on at least three arrays and interquartile range (IQR) > 0.2 on at least 5 probes per gene on the array were selected for further analysis. The top 1000 most variable genes were used for principal component analysis (PCA) using MultiExperiment Viewer version 4.8.4. Signal log2 ratios, which represent fold changes (FC), and related significances of change were calculated from the mean intensities of the control and high sFlt-1 group using intensity-based moderated t statistics (IBMT) implementing Bayes correction [16]. Resulting log2 ratios and p values were applied for further analysis of the data. Gene set enrichment analysis was used to relate the microarray data to networks of diseases and biological functions. Heat maps generation showing a comparison of gene expression patterns was done with MultiExperiment Viewer version 4.8.4.

Genomic DNA isolation and pyrosequencing

Fetal livers were homogenized with a TissueLyser LT (Qiagen, Hilden, Germany) for 1 minute at 40 Hz. Genomic DNA was isolated using allprep DNA/RNA mini kit (Qiagen, Venlo, the Netherlands), following manufacturer’s protocol. DNA purity and concentration was validated on Nanodrop 2000c (Thermo Scientific, Pittsburgh PA, USA), while 500 ng of genomic DNA was used for bisulfite conversion with the EZ DNA Methylation Gold Kit (Zymo Research, Leiden, the Netherlands) according to manufacturer’s protocol. Samples were stored at -20°C until analysis. With Pyromark Assay Design 2.0 software (Qiagen), bisulfite specific primers were designed for the rat Ppar alpha promoter region (forward: 5’-biotin-GAGGTTAGGTGGGAAGTTTTATTAG-3’; reverse: 5’-AACCTAAAAACCTAAACAATTCT-3’). Twenty nanograms of bisulfite-modified DNA were used for amplification with HotStarTaq master mix using the following steps: DNA polymerase activation (95°C for 15 min), three-step cycle of denaturation (94°C for 30 sec), annealing (58°C for 1 min) and extension (72°C for 45 sec) repeated for 45 cycles in a row. Finally, the last extension was carried out at 72°C for 7 minutes. The PCR product was analyzed using the sequencing primer: 5’-CTAAATTTTTACTACCCAAAAT-3’ and sequence to analyze: CACCCRACRA ACRAAAAAC ACCTAAAC AAAACRCRCT AACRAAARC RCTTTTCTAC TCCAACCRC RCRACCR, for the extent of methylation per selected CpG position by pyrosequencing using a Pyromark Q24 system and analysis software 2.0 (Qiagen). The level of DNA methylation is given as a percentage.
Statistical analysis

All data are presented as mean ± SEM. Differences between groups were calculated with a unpaired t-test (two groups) or one-way ANOVA (three groups). Comparison of multiple groups (DNA methylation analysis of different CpG positions) was analyzed by two-way analysis of variance (ANOVA), followed by a Bonferroni posthoc test. For all statistical tests, a p-value < 0.05 was considered significant. All data were analyzed using GraphPad Prism 6.0 Software (GraphPad).

Results

Maternal characteristics upon sFlt-1 overexpression

To validate our overexpression model we measured concentrations of sFlt-1 in plasma, 96 hours post adenovirus injections (gestational day 12) and at gestational day (GD) 19. Surprisingly, plasma sFlt-1 concentrations in the overexpressed animals were not in the same range. We identified animals that have moderate increase (n=4) of sFlt-1 concentrations in plasma at GD12 (mean 310 ng/ml ± 109) and GD19 (mean 640 ng/ml ± 624) (Figure 1A), and animals (n=3) with high concentrations of plasma sFlt-1 in the same time points (mean GD12 1073 ng/ml ± 735; mean GD19: 4726 ng/ml ± 2215) (Figure 1A). In that way, we characterized two groups of dams with low and high sFlt-1 concentrations in addition to the control group, where the sFlt-1 concentrations at GD19 were almost zero (mean 0.08 ng/ml ± 0.008). In addition, the aortic blood pressure was assessed at GD19 and there were no significant differences between the control and low sFlt-1 group. In comparison, the high sFlt-1 group showed decreased diastolic and mean arterial pressure (Figure 1B). Furthermore, excessive proteinuria was not present in any of inspected groups (Figure 1C).

![Figure 1](image)

Figure 1. Overexpression of sFlt-1 in pregnant dams. (A) plasma sFlt-1 concentrations in dams (n=3-4), (B) Blood pressure values (*p<0.05, n=3-4), (C) Protein levels in 24 hours urine from pregnant dams (n=3-4). SAP=systolic arterial pressure, DAP=diastolic arterial pressure, MAP=middle arterial pressure.
Fetuses from dams that have high sFlt-1 concentrations are growth restricted

It was reported that increased sFlt-1 concentrations in the mother can result in growth restriction of the fetus [15]. In our study, fetuses that were exposed to high sFlt-1 concentrations were approximately 22% lighter and the length was reduced by 9% in comparison to controls (Figure 2A, B). Also, the body proportionality, as expressed by the ponderal index PI (weight in grams/length in mm), was lower in the fetuses exposed to high sFlt-1 concentrations when compared to the fetuses exposed to low or no sFlt-1 (Figure 2C). There were no differences between these parameters in the control and the low sFlt-1 group (Figure 2).

Figure 2. Fetal characterization. (A) Fetal body weight at gestational day 19 (n=10), (B) Fetal crown-rump length at gestational day 19 (n=10), (C) Fetal weight to length ponderal index (n=10). ***p<0.001.

Brain-sparing and increased sFlt-1 concentrations in fetuses exposed to high sFlt-1 maternal concentrations

To evaluate whether the fetal growth restriction is asymmetrical or symmetrical we assessed the organ weight at GD 19. The liver weights of fetuses exposed to high maternal sFlt-1 concentrations were smaller in comparison to the control group (Figure 3A). Brain weight was uncompromised in all groups (Figure 3B). We also checked the brain/liver ratio as an indicator of fetal asymmetrical/symmetrical growth. We observed increased brain/liver ratio in the fetuses exposed to high sFlt-1 as compared with the low sFlt-1 and the control group and no changes in the low sFlt-1 group as compared with the control group (Figure 3C), indicating that brain-sparing occurs in the fetuses exposed to the high sFlt-1. To evaluate whether the maternal sFlt-1 levels could influence the fetal sFlt-1 levels, we measured the plasma sFlt-1 concentrations in the fetuses. We observed that fetuses exposed to high sFlt-1 during pregnancy have an 18-fold increase of sFlt-1 in fetal plasma in comparison to control and low sFlt-1 group (Figure 3D).
Figure 3. Asymmetrical growth restriction in fetuses exposed to high sFlt-1. (A) Fetal liver weight as percentage of body weight at gestational day 19 (n=10), (B) Fetal brain weight as percentage of body weight at gestational day 19 (n=10), (C) Brain to liver ratio (n=10), (D) Fetal plasma sFlt-1 concentrations at gestational day 19 (n=10). **p<0.01, ***p<0.001.

sFlt-1 overexpression and growth restriction modulate the fetal liver gene expression

In order to detect whether the fetal growth restriction due to the sFlt-1 overexpression affected the liver function, we performed microarray analysis on fetal liver RNA. We performed an unsupervised clustering analysis on the top 1000 most variable genes based on interquartile range (IQR), which is an indicator of variability of gene expression. In continuation, we performed principal component analysis (PCA) of the top 1000 most variable genes. As shown in Figure 4A the samples tend to cluster in two separate clusters in principle component 1 (PC1), which accounts for 34 % of the gene expression. Only one fetal liver from the high sFlt-1 group tends to cluster with the control ones. The dendrogram plot, accompanied by a heat map of the gene expression differences between the groups is presented in Figure 4B. As expected, the liver gene expression profiles between the groups were recognizably different meaning that exposure to high sFlt-1 concentrations in utero can mediate fetal liver molecular changes. Further analysis depicted that there is upregulation of several genes involved in the fatty
acid beta-oxidation, including the master regulators such as Cpt1a, Cpt1b, and Acadvl (Figure 4C).

**Figure 4.** Analysis of the transcriptional response to high sFlt-1 in fetal liver. (A) principal component analysis on the top 1000 differentially expressed genes. (B) heatmap showing top 1000 differentially expressed genes in the liver in response to sFlt-1. (C) heatmap showing enriched fatty acid beta-oxidation gene set.

**Fatty acid metabolic pathways are upregulated in liver from growth-restricted fetuses exposed to high sFlt-1 concentrations**

Since many genes were upregulated in the high sFlt-1 fetal livers as compared to the control fetal livers, we performed a GSEA in order to evaluate the pathways in which these genes are involved. We used gene set enrichment analysis (GSEA) to analyze GO and KEGG gene sets that are significantly altered in the livers from the high sFlt-1 group in comparison to the controls. Top downregulated pathways involved olfactory sensing pathways and g-gated potassium and calcium channels (Supplementary Table 1). Moreover, significantly enriched metabolic pathways included genes responsible for fatty acid beta-oxidation; fatty acid, triacylglycerol, and ketone body metabolism. In addition,
gene set enrichment analysis indicated that genes involved in the \textit{Ppara} signaling pathway are induced upon sFlt-1 overexpression (\textbf{Table 1}).

\textbf{Table 1.} Gene set enrichment analysis of upregulated pathways in response to high sFlt-1.

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\textit{Ppara} is differentially methylated in fetal liver upon fetal growth restriction due to sFlt-1 overexpression

Moreover, it was reported that protein-restricted diet during pregnancy influences the methylation rates of the \textit{Ppara} promoter region in livers from juvenile offspring [17]. To investigate whether the methylation status of \textit{Ppara} is influenced in our growth restricted and sFlt-1 exposed fetuses, we designed a rat specific pyrosequencing assay for \textit{Ppara} that encompasses 10 CpG positions located 500 bp upstream the transcriptional start site (TSS) (\textbf{Figure 5A}). Methylation of CpG position 5 was decreased in the high sFlt-1 group (\textbf{Figure 5B}) and the average methylation of the selected region showed hypomethylation (\textbf{Figure 5C}), which is in accordance with the previously reported results.
Figure 5. Ppara is differentially methylated in the liver in response to high sFlt-1. (A) Schematic representation of promoter region of rat Ppara. (B) Methylation percentage levels per each inspected CpG site (n=6, two-way analysis of variance test). (C) Average methylation percentage per inspected area (n=6), *p<0.05.

Discussion

The results of the present study indicate that maternal sFlt-1 overexpression has an important implication in the fetal liver physiology. Despite the limited changes in the maternal physiology, exposure to a high sFlt-1 concentration from middle to the end of gestation resulted in fetal growth restriction. This was accompanied by decreased liver weight, altered gene expression and Ppara methylation in the fetal liver.

It was reported that sFlt-1 overexpression during pregnancy contributes to the pathophysiology of preeclampsia [15,18]. However, in our study, despite the increased viral load to induce sFlt-1 overexpression, we could not record the clinical symptoms of preeclampsia. One possible explanation could be that our model produced either too high or too low sFlt-1 concentration instead of the middle range concentrations that were obtained with other models [15,18,19]. It can be speculated that these conflicting results
are due to the differences in the timing and methods used to track the blood pressure and proteinuria. The advantage of this specific model is that we can study the effects of high levels of sFlt-1 on the fetuses in the absence of maternal signs, such as hypertension and proteinuria. Therefore, we consider our models as a model of sFlt-1 overexpression.

Using this maternal sFlt-1 overexpression model, we demonstrate that the increased maternal sFlt-1 concentrations lead to asymmetrical growth restriction of the fetus. This is a condition where the brain development is preserved at the expense of liver growth. The brain sparing effect usually occurs due to maternal and/or fetal hypoxia where concomitant redistribution of the oxygenated blood is established at the expense of other organs [20,21]. In addition, brain/liver ratio is an important predictor of the fetal outcome [22]. It is well established that redistribution of nutrients leads to a reduction of the functional capacity of metabolically active organs e.g. liver. This adaptation can lead to metabolic programming and a thrifty phenotype that can persist later in life. Several studies, previously have shown that low birth weight increases the risk of cardiovascular and metabolic diseases e.g. stroke, diabetes, obesity etc [23–27]. Potential factors include malnutrition and toxins present during pregnancy. Our data demonstrate that fetal exposure to sFlt-1 is associated with asymmetrical growth restriction, however to what extent this contributes to developmental programming of the fetus still remains to be determined.

Moreover, we observed that maternal plasma sFlt-1 concentrations result in increased plasma sFlt-1 concentrations in the fetuses as well. These results support earlier observations, that angiogenic factors are increased in maternal and umbilical cord samples in pregnancies complicated with intrauterine growth restriction [28]. Additionally, several other studies have reported that newborns small for gestational age (SGA) also have increased sFlt-1 concentrations in umbilical plasma samples [29,30]. These results suggest that the fetuses are not only exposed to the indirect effects of sFlt-1 but also via direct exposition to sFlt-1. It is possible that sFlt-1 interferes with the fetal organ angiogenesis, but the exact mechanism will require further investigation.

Importantly, we observed that the gene expression profile is changed in fetal livers that were exposed to high sFlt-1 concentrations in utero. This difference was prominent in the genes involved in the fatty acid metabolism, which is crucial for the energy supply of the fast growing fetal organism. This is in accordance with a mouse study of sFlt-1 overexpression where it was found that LXR/RXR pathway was the top modified canonical pathway in the plasma proteome of dams 6 months postpartum [13].
pathway is involved in the regulation of the lipid metabolism. In contrast, a calory restricted diet results in decreased expression of genes involved in fatty acid synthesis [31] and folate-restricted diet increases the expression of genes involved in fatty acid degradation and beta-oxidation processes [32]. These findings suggest that expression modulation of genes involved in fatty acid metabolism serves as a compensatory mechanism in fetal growth restriction, obtained either via nutritional or via antiangiogenic manipulation.

In agreement with the upregulated fatty acid metabolism genes, we observed that the Ppara targets pathway was enriched as well. Ppar genes are a unique set of fatty acid-regulated transcriptional factors that increase fatty acid metabolism [33]. In particular, Ppara acts as a key nutritional and environmental sensor in order to establish metabolic adaptation [34]. Activation of Ppara induces uptake and catabolism of fatty acids by upregulation of genes involved in beta-oxidation and fatty acid transport [35]. Moreover, the peroxisome proliferator-activated receptors (PPARs) have a substantial role in fetal metabolism and are well known for their role in developmental programming [36].

It was reported that fetal growth restriction due to a caloric restricted maternal diet results in decreased Ppara expression and can be maintained for longer period of time [37]. However, growth restricted livers from protein-restricted mothers [17] have increased Ppara expression that is in accordance with our results. It appears that the differences in the fatty acid metabolism and Ppara signaling are also affected in our sFlt-1 exposed growth-restricted fetuses.

We found moderate but significant changes in the promoter methylation pattern of Ppara in the fetal liver exposed to the highest sFlt-1 concentrations in utero. Previously, it was reported that Ppara is differentially methylated in the offspring livers of growth-restricted rats exposed to low protein and calorie restriction intake in utero. In continuation, it was shown that these discrete changes are stable and persistent in adulthood[17]. These Ppara methylation differences in early life might be considered as an indicator of metabolic adaptation to the intrauterine harsh environment.

Together, these results show that high maternal levels of sFlt-1 result in fetal growth restriction, high fetal plasma sFlt-1 levels, changes in fatty acid gene expression and their master regulator Ppara, that also shows promoter methylation alterations in accordance with the gene expression. This is consistent with other models of
developmental programming e.g. protein restriction [17]. These findings should warrant further investigations into the sFlt-1 contribution to the developmental programming of offspring health.
References


Fetal programming in pregnancy-associated disorders


[27] Von Beckerath AK, Kollmann M, Rotky-Fast C, Karpf E, Lang U, Klaritsch P. Perinatal complications and
Fetal programming in pregnancy-associated disorders


**Supplementary table 1.** Gene set enrichment analysis of downregulated pathways in response to high sFlt-1.

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