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# Mitochondrial DNA content and methylation in fetal cord blood of pregnancies with placental insufficiency

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ABSTRACT

*Introduction:* Intrauterine growth restriction (IUGR) and preeclampsia (PE) are pregnancy disorders characterized by placental insufficiency with oxygen/nutrient restriction and oxidative stress, all influencing mitochondria functionality and number. Moreover, IUGR and PE fetuses are predisposed to diseases later in life, and this might occur through epigenetic alterations.
Here we analyze content and methylation of mitochondrial DNA (mtDNA), for the first time in IUGR and PE singleton fetuses, to identify possible alterations in mtDNA levels and/or epigenetic control of mitochondrial *loci* relevant to replication (*D-loop*) and functionality (mt-*TF/RNR1*: protein synthesis, mt-*CO1*: respiratory chain complex).
*Methods:* We studied 35 term and 8 preterm control, 31 IUGR, 17 PE/IUGR and 17 PE human singleton pregnancies with elective cesarean delivery. Fetal cord blood was collected and evaluated for biochemical parameters. Extracted DNA was subjected to Real-time PCR to assess mtDNA content and analyzed for *D-loop*, mt-*TF/RNR1* and mt-*CO1* methylation by bisulfite conversion and pyrosequencing.
*Results:* mtDNA levels were increased in all pathologic groups compared to controls. Mitochondrial *loci* showed very low methylation levels in all samples; *D-loop* methylation was further decreased in the most severe cases and associated to umbilical vein $pO_2$. mt-*CO1* methylation levels inversely correlated to mtDNA content.
*Discussion:* Increased mtDNA levels in IUGR, PE/IUGR and PE cord blood may denote a fetal response to placental insufficiency. Hypomethylation of *D-loop*, mt-*TF/RNR1* and mt-*CO1 loci* confirms their relevance in pregnancy.



## 1. Introduction

Intrauterine growth restriction (IUGR) and preeclampsia (PE) are pregnancy disorders characterized by defective placental functions, leading to impaired oxygen and nutrients transfer to the fetus [1–5], and increased oxidative stress and inflammation [6]. Adverse intrauterine conditions are known to have an impact also on adult health of newborns, predisposing them to later pathologies such as diabetes, cardiovascular diseases and allergic sensitization [7–9]. Reprogramming of fetal epigenome by intrauterine exposures can occur through methylation of DNA, affecting gene expression and activity without changes in DNA sequence.

Mitochondria, as cell energy producers, have been recently investigated as potentially associated with the pathogenesis of placental insufficiency. The number of mitochondria is proportional to the energy requirements of the cells and can deviate from a "healthy range" in conditions of altered oxygen/nutrients availability or oxidative stress impairing mitochondrial functionality [10].

Mitochondria have their own DNA, coding for respiratory chain enzymes, which is distinct but in continuous cross-talk with the nuclear genome. The amount of mitochondrial DNA (mtDNA) is recognized as a measure of the mitochondrial content [11]. Changes

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**Abbreviations**

| | |
|---|---|
| IUGR | intrauterine growth restriction |
| PE | preeclampsia |
| mtDNA | mitochondrial DNA |
| CO1 | Cytochrome C Oxidase I |
| TF/RNR1 | tRNA Phenylalanine/12S RNA |
| $pO_2$ | oxygen partial pressure |
| BMI | Body Mass Index |

in mitochondrial DNA levels have been consistently reported in placenta and maternal blood of pathologic pregnancies [12–17]. However, no data are available about mtDNA content in fetal blood of IUGR and PE singleton pregnancies.

Mitochondrial DNA, in addition to nuclear DNA, is subjected to cytosine methylation by a mitochondrial-specific DNA methyltransferase [18]. Methylation makes DNA less accessible to replication and transcription, therefore it may potentially interfere with the expression of respiratory chain complexes, impacting on mitochondrial functionality. Few studies have been conducted on mtDNA methylation, focusing on degenerative diseases, cancer, aging and exposition to environmental pollutants [19–24].

In this study, we investigated mitochondrial DNA in fetal cord blood of pregnancies affected by IUGR and/or PE. In particular, we evaluated whether alterations of mitochondrial content, reported for placentas and maternal blood, are also present in the fetus, and we analyzed the methylation levels of three mitochondrial genes in pathologic *versus* control fetuses to evaluate for a possible epigenetic control of mitochondrial number and gene expression. *D-loop*, mt-*CO1* and mt-*TF/RNR1* are mitochondrial *loci* relevant to mtDNA and mitochondrial functionality that have been already tested in methylation studies on other pathologies [19–24]. *D-loop* control region is involved in mtDNA replication, mt-*TF/RNR1 locus* contains two genes respectively constituting phenylalanine tRNA (*TF*) and 12S rRNA (*RNR1*), both needed for protein synthesis, whereas mt-*CO1* encodes for Cytochrome C oxidase subunit 1, belonging to respiratory chain and thus involved in mitochondrial function.

## 2. Methods

### 2.1. Population

One hundred and eight pregnancies were studied: control pregnancies at term (n = 35) and preterm (n = 8), and pregnancies complicated by placental insufficiency (IUGR: n = 31; PE/IUGR: n = 17; PE: n = 17).

Only patients with singleton pregnancies undergoing elective Cesarean section were included in this study. Exclusion criteria for all groups were maternal drug or alcohol abuse, maternal or fetal infections, fetal abnormal karyotype or major malformations. All pregnant women were of Caucasian origin.

Controls were term (>37 weeks) or preterm (≤37 weeks) pregnancies with normal intrauterine growth and appropriate-for-gestational-age birth weight according to reference ranges for the Italian population [25]. Indications for Cesarean section were breech presentation, previous Cesarean delivery or maternal indications not influencing fetal growth.

IUGR fetuses were identified *in utero*, through longitudinal measurements indicating abdominal circumferences below the 10$^{th}$ percentile of age-related reference values and a shift from the reference growth curve greater than 40 centiles [26]. IUGR pregnancies were further classified according to umbilical artery pulsatility index, measured by Doppler velocimetry [27–29].

Preeclampsia was defined as blood pressure >140/90 mmHg in two measurements/24 h and proteinuria >300 mg/24 h after the 20$^{th}$ week of pregnancy in a previously normotensive and non-proteinuric woman [30]. PE pregnancies were further divided in two subgroups, with disease onset before or after the 34$^{th}$ week of gestation.

The study was approved by the Institutional Ethics Committee, and all pregnant patients gave their informed consent.

### 2.2. Sampling

Umbilical blood was collected from a doubly-clamped segment of the cord at the time of Cesarean section and stored at −20°C until analysis.

Oxygenation and acid-base parameters of umbilical artery and vein blood were measured immediately after delivery using a GEM Premier 3000 portable system (Instrumentation Laboratory).

### 2.3. mtDNA analysis

Total DNA was extracted from cord blood samples using QIAamp DNA Blood Mini Kit (Qiagen; Valencia, CA, USA) and quantified by NanoDrop ND 1000 spectrophotometer (NanoDrop Technologies; Wilmington, DE, USA).

mtDNA content was assessed in Real-time PCR experiments by normalizing the levels of a mitochondrial gene (*Cytochrome B*) to those of a single-copy nuclear gene (*RNase P*). For each gene, 30 ng of total DNA were analyzed in triplicate with TaqMan assays (Hs02596867_s1 and 4316849) on the 7500 Fast Real-Time PCR System (Applied Biosystems by ThermoFisher Scientific; Carlsbad, CA, USA). Cq values with standard deviation exceeding 0.25 were excluded and experiments repeated. The median inter-run coefficient of variation was 1.90%. For each sample, mtDNA level was calculated as $2^{-\Delta Cq}$, obtained after subtracting *RNase P* average Cq value to *Cytochrome B* average Cq value (ΔCq).

mtDNA methylation analyses were performed in a subset of cord blood samples (24 term controls, 6 preterm controls, 24 IUGR, 14 PE/IUGR and 9 PE).

Total DNA samples (100–500 ng) were bisulfite-converted using EZ DNA Methylation-Direct Kit (Zymo Research Corporation; Irvine, CA, USA) and eluted in 30 μl of M-Elution buffer.

Bisulfite-converted DNA (20–50 ng) was subjected to PCR of mitochondrial *D-loop*, *TF/RNR1* and *CO1* segments, in a final volume of 50 μl, with GoTaq Hot Start Polymerase (Promega; Madison, WI, USA) and specific primers (Supplementary Table). Cytosine methylation was quantified by pyrosequencing using primers described in Supplementary Table and PyroGold SQA Reagent Kit (Qiagen). Pyrosequencing also allowed to verify bisulfite conversion occurred properly: data from incompletely converted samples were excluded and experiments repeated. The methylation percentage at each CpG site was quantitatively analyzed by PyroMark ID instrument and software Q-CpG v.1.0.11 (both Qiagen). Methylation values represent the mean between at least two independent PCR and pyrosequencing experiments, with a standard deviation ≤3%. The median inter-run coefficient of variation was 8.08%.

Real-time PCR and pyrosequencing runs were carried out in a blinded and randomized fashion.

### 2.4. Statistical analysis

Data distribution was evaluated with the Kolmogorov-Smirnov test. Maternal age and *D-loop* methylation levels, showing normal