TITLE:

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

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KEY WORDS

mitochondria; methylation; cord blood; intrauterine growth restriction; preeclampsia

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.

ABBREVIATIONS

IUGR: intrauterine growth restriction

PE: preeclampsia

mtDNA: mitochondrial DNA

CO1: Cytochrome C Oxidase I

TF/RNR1: tRNA Phenylalanine / 12S RNA

BMI: body mass index

pO_2: oxygen partial pressure

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 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.

METHODS

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 10th 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/24h and proteinuria >300mg/24h after the 20th week of pregnancy in a previously normotensive and nonproteinuric woman [30]. PE pregnancies were further divided in two subgroups, with disease onset before or after the 34th week of gestation.

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

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).

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.

Statistical analysis

Data distribution was evaluated with the Kolmogorov-Smirnov test. Maternal age and *D-loop* methylation levels, showing normal distribution, were compared between two groups using independent-samples t-test, with applied correction when the equality of variances assumption was violated (Levene's test). All other clinical and molecular data were analyzed by independent-samples Mann-Whitney U test. Correlation between values was assessed using bivariate Pearson correlation and the r coefficient reported. Differences and correlations were considered statistically significant when $p<0.05$. No adjustments for multiple comparisons were made.

Analyses were performed using the statistical package SPSS (IBM SPSS Statistics, v.23; Armonk, NY, USA).

RESULTS

Clinical data of the study population

Maternal and fetal characteristics of cases and controls are compared in Table 1.

As expected, PE women had higher pre-pregnancy BMI. All pathologic cases had lower gestational age, placental and fetal weight than term controls. However, gestational age of IUGR and PE fetuses was similar to the preterm control subgroup.

mtDNA content

No significant relationship was observed between mtDNA content and gestational age in control pregnancies. Moreover, there were no significant differences between term and preterm controls (Figure 1A). Based on these observations and given the small size of the preterm control group, we pooled the term and preterm controls for all subsequent comparisons.

All cases presented a strong significant increase in mtDNA levels compared to controls (Figure 1B; IUGR $p=0.000$; PE/IUGR $p=0.004$; PE $p=0.000$).

We further examined pathologic samples after classifying them for disease severity: IUGR (with or without PE) were divided in two subgroups with normal or altered pulsatility index, PE pregnancies with disease onset before or after the 34th week of gestation. As shown in Figure 2, mtDNA content was significantly increased in all cases, independently from their severity, and no differences were observed within IUGR or PE groups.

mtDNA methylation

We then further analyzed mitochondrial DNA by evaluating, in a subset of cord blood samples, methylation levels of three mitochondrial segments, *D-loop*, *CO1* and *TF/RNR1*, involved in mitochondrial replication and function. In this sample subset, clinical characteristics and the relative number of cases and controls were similar to the entire population.

No significant relationship was observed between methylation levels and gestational age in control pregnancies, nor significant differences between term and preterm controls (Figure 3A), in any analyzed mitochondrial region.

Both cases and controls displayed low percentages of methylated cytosines (Figure 3B), pointing out a shared hypomethylation pattern in all cord blood samples (*D-loop*: 0.55-10.75 %; mt-*CO1*: 1-8.5 %; mt-*TF/RNR1*: 0-14.75 %). Moreover, *D-loop* methylation levels were significantly decreased in PE/IUGR compared to controls (p=0.04; Figure 3B). Supplementary Figure 1 shows *D-loop* methylation levels of each investigated CpG for cases and controls.

We then re-analyzed *D-loop* data after classifying pathologic cases according to their severity. The most severe cases, both in IUGR and in PE, displayed a significant reduction of *D-loop* methylation compared to controls (Figure 4), even greater than what observed for the PE/IUGR group *versus* controls (Figure 3B). Conversely, mild IUGR and PE samples did not show significant differences.

D-loop, mt-*CO1* and mt-*TF/RNR1* methylation levels displayed a significant positive correlation with each other (Supplementary Figure 2).

Methylated cytosines (%) in *D-loop* region were significantly related to umbilical vein oxygen partial pressure (pO_2; Figure 5A). A significant relationship was also observed between *D-loop* methylation and both gestational age and fetal weight (Figures 5B and 5C).

Finally, we found a significant and inverse correlation between mt-*CO1* methylation levels and mtDNA content, both in pathologic samples (r=-0.431, p=0.014; data not shown) and in the whole population (r=-0.369, p=0.006; Figure 5D).

DISCUSSION

Here we analyzed the mitochondrial DNA amount in fetal blood from pregnancies characterized by IUGR or preeclampsia compared to control pregnancies. We found significantly higher mtDNA levels in all pathologic groups than in controls, which included also preterm pregnancies with gestational age similar to cases. These results are consistent with a previous study comparing cord blood mtDNA in monochorionic twins with one IUGR fetus, that reported higher mtDNA amount in the IUGR fetuses than in their respective larger twins [31]. Moreover, our findings give novel insights into preeclamptic pregnancies, being to our knowledge the first mtDNA data in PE cord blood to be described.

We previously reported that mtDNA levels, accounting for mitochondrial content, are increased in IUGR placental tissue and placental mesenchymal stromal cells but decreased in cytotrophoblast cells, compared to controls, suggesting different mitochondrial contents depending on the analyzed cell lineages [12,13,32]. A different cell composition may indeed account for the lower mtDNA amount conversely found in IUGR placentas in another study [14]. Increased mtDNA levels were also reported in blood of women with IUGR [16] and PE [17] pregnancies.

Our present results suggest that fetuses react to placental insufficiency and to the adverse intrauterine environment by increasing their mitochondrial content, with a compensatory mechanism facing the oxidative stress and/or hypoxia and calorie restriction occurring in pathologies related to placental insufficiency [33,1]. In particular, IUGR fetuses are characterized by oxygen and nutrient restriction, which is known to induce mitochondrial biogenesis [34]. Preeclamptic pregnancies are instead characterized by increased oxidative stress, reported in placenta, mothers and fetuses [35-36] where it might damage mitochondria, thus inducing their biogenesis.

Given the recent demonstration of mitochondrial DNA methylation [18] and the known impact of adverse intrauterine conditions on epigenetic modifications, we analyzed methylation of important mitochondrial genes in cord blood of fetuses grown in an impaired environment. We focused on three mitochondrial *loci*, relevant to mtDNA and mitochondrial functionality.

The first result of our analysis is the common hypomethylation pattern shared by all cord blood samples (controls and pathologic pregnancies), with percentages of methylated cytosines below 15%. Low methylation levels had already been reported for two of these *loci*, *D-loop* (mean %: 4.0) and mt-*RNR1* (mean %: 11.7), in cord blood of fetuses from the ENVIRO*N*AGE birth cohort, which includes a very heterogeneous population but predominantly composed by normal pregnancies with vaginal deliveries [37]. This study [37] also reports a wide variability for mt-*RNR1* methylation, similar to our mt-*TF/RNR1* methylation data in control samples, which could however be partially explained by assay sensitivity. The observed hypomethylation, making mtDNA accessible to replication and transcription with the potential to be expressed, confirms that these mitochondrial *loci* and their products are essential and required in any pregnancy condition.

Nevertheless, we found further decreased *D-loop* methylation in the most severe cases, namely PE/IUGR (14 cases), early-onset PE (3 cases) and IUGR with altered umbilical artery pulsatility index (15 cases). Moreover, low *D-loop* methylation levels were associated to poorer fetal outcomes, as indicated by their significant positive correlation with gestational age, fetal weight and umbilical vein pO_2, all reflecting fetal conditions, in pathologic cases. A similar negative association between *D-loop* methylation levels and disease severity was reported in colorectal cancer [21].

The *D-loop* region controls mtDNA replication and its hypomethylation makes it more accessible to replication machinery. Although the mild decrease we report might not be functionally meaningful, we can hypothesize that it may partially explain the increased mtDNA content we found in pathologic samples. However, additional factors can mediate mitochondrial DNA replication, such as POLG DNA polymerase or helicases [38], and their alterations would probably contribute to mtDNA increase.

In addition, we found a significant negative correlation between mt-*CO1* methylation and mtDNA content.

Limitations

One possible bias for the analysis of IUGR and PE pregnancies in control/case studies is the different gestational age, as in pathologic cases delivery is frequently induced earlier to preserve fetal and maternal health. In our study we were able to include 8 preterm control pregnancies, with normal intrauterine growth and gestational age similar to cases. This type of population is very uncommon, as preterm deliveries are often associated with fetal growth-related pathologies, thus we were not able to have a larger group, and this may represent a limitation. Of note, in cord blood of preterm fetuses we did not find any significant difference compared to term controls, in either mitochondrial DNA content or methylation, nor we observed significant correlations of these molecular data with gestational age in the control population. We may thus hypothesize that both mtDNA content and methylation are independent from gestational age in our population.

Mitochondrial DNA in blood derives from white blood cells (that also bear nuclear DNA), platelets, microvescicles and cell-free DNA. Since platelets and microvescicles do not contain nuclear genome, an increase in their number would be reflected in higher abundance of mtDNA in whole blood. Unfortunately, we did not have hematocrit data or platelet indices for our population, thus we cannot exclude a different blood composition in our samples. Nevertheless, newborns from PE pregnancies or weighting less than 10^{th} centile often present lower platelet number [39-40] and increasing platelet counts were found with advancing gestational age [41]. We can thus hypothesize that the increased mtDNA levels we observed were not due to higher platelet counts in pathologic cord blood, although we cannot totally exclude this limit.

Conclusions

In this study we describe increased mitochondrial content in fetal blood of IUGR, PE/IUGR and PE pregnancies, suggesting a fetal response to restricted nutrients and oxygen availability as well as to oxidative stress.

Moreover, this is the first study to our knowledge investigating DNA methylation of important mitochondrial regions in cord blood of pregnancies with placental insufficiency. We found a common hypomethylation pattern shared by both controls and pathologic cases, indicating the relevance of these mitochondrial genes (*D-loop*, *CO1*, *TF/RNR1*) that need to be expressed. Future analyses, e.g. investigating mitochondrial gene expression and function, are needed to further explore these hypotheses and to identify the relative contributions of cord blood cells or cell-free DNA to the observed results.

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DISCLOSURE STATEMENT

The authors report no conflict of interest.

	TERM CONTROLS	PRETERM CONTROLS	IUGR	PE/IUGR	PE
	(n = 35)	(n = 8)	(n = 31)	(n = 17)	(n = 17)
Maternal age, years	34 (19-39)	36 (27-40)	35 (22-45)	36 (33-43) *	37 (17-44)
Pre-pregnancy BMI, kg/m^2	21.2 (18.7-25.2)	20.2 (19.3-31.3)	20.2 (17.2-32.1)	23.2 (17.5-37.7) *	22.8 (18-35.6) *
Gestational age, weeks	39.0 (37.6-40)	36.0 (31.7-37) ***	35.9 (28.4-39.7) ***	33 (26.7-37.4) ***$^{+}$	34.6 (27.7-38.9) ***
Fetal weight (F), g	3200 (2630-3920)	2745 (2580-3300) **	1820 (500-2620) ***$^{+++}$	1230 (660-2250) ***$^{+++}$	2020 (800-3170) ***$^{+}$
Placental weight (P), g	670 (415-950)	600 (520-750)	273 (120-580) ***$^{+++}$	215 (113-378) ***$^{+++}$	350 (135-700) ***$^{++}$
F/P weight ratio	4.84 (3.37-6.63)	4.62 (3.49-5.31)	5.71 (3.32-10.75) *	5.56 (3.3-12.3)	5.52 (2.43-9.45)

Table 1. Maternal, fetal and placental data of cases and controls. Data are presented as median and range. BMI: Body Mass Index. *p<0.05, **p<0.01, ***p<0.001 *versus* term controls; $^{+}$p<0.05, $^{++}$p<0.01, $^{+++}$p<0.001 *versus* preterm controls.

Gene	Position in the mtDNA	PCR primers 5' → 3'	Amplicon size / Annealing Temperature	Pyrosequencing primers 5' → 3'	Sequence to analyze
TF/RNR1	597 – 765	**F:** TAAAGTAATATATT GAAAATGTTTAGA **R(bio):** TACTTAATACTTAT CCCTTTTAATC	168 bp / 52°C	**Seq1:** TATTGAAAATGTTTAGA **Seq2:** GATTATATATGTAAGTA TTT	**Seq1:** YGGGTTTATATT **Seq2:** TYGTTTTAGTGAGTTTATTTTTTA AATTATTAYGA
D-loop	6 – 259	**F:** TGTGTAGATATTTA ATTGTTATTATTA **R(bio):** CAAATCTATCACCC TATTAACCAC	253 bp / 54°C	**Seq1:** TATTTTAGTAAGTATGT **Seq2:** TATTGTGATATAGGGT	**Seq1:** TYGTTTGTAATATTGAATGTAGG TGYGAT **Seq2:** GTTTYGGTTTTAGYGTTTYGTAA TGTTATYGYGTGTAT
CO1	5882 – 5999	**F(bio):** TATTTTATTTTATTT TTATTGATGT **R:** AACTATACCTAAAA CTCCAACTCA	117 bp / 54°C	TAAAACTCCAACTCATA	CRCCRAATAATAAATATAATATT CCAATATCTTTATAATTTATAAA AAATAATCAACRATCRACRA

Supplementary Table. PCR and pyrosequencing primers and conditions. F: forward, R: reverse, (bio): biotin-labeled. Analyzed CpGs are underlined.

FIGURES

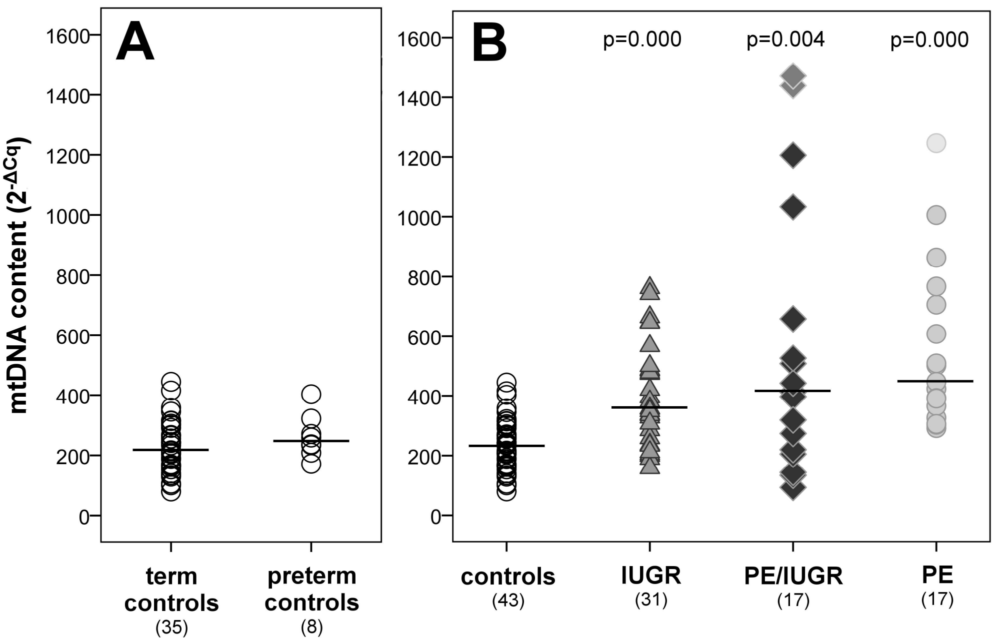


Figure 1: mtDNA levels in cord blood of (**A**) term and preterm controls, (**B**) control (term+preterm), IUGR, PE/IUGR and PE pregnancies. Medians and significant p values *versus* controls are displayed. mtDNA content was calculated as $2^{-\Delta Cq}$, where ΔCq = *Cytochrome B* (mt gene) Cq – *RNase P* (nuclear gene) Cq.

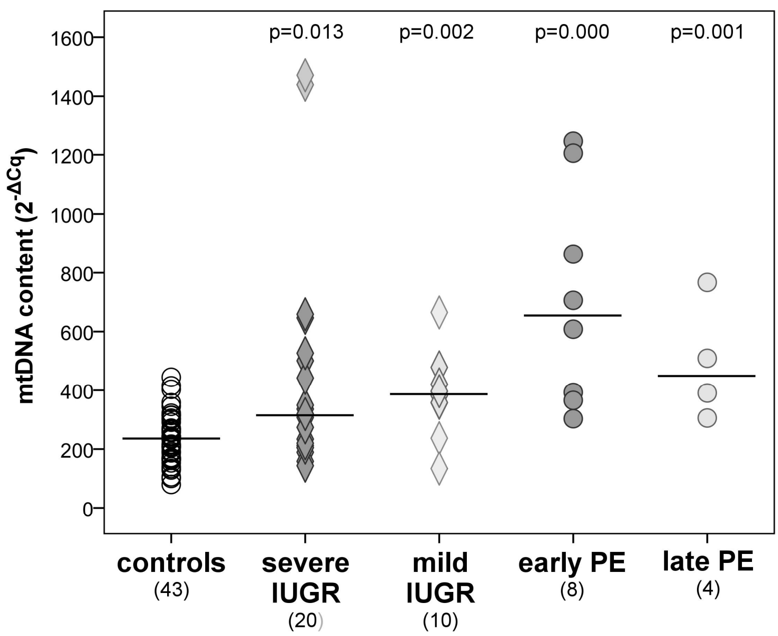


Figure 2: mtDNA content in pathologic samples divided according to severity. Medians and significant p values *versus* controls are displayed. mtDNA content was calculated as $2^{-\Delta Cq}$, where ΔCq = *Cytochrome B* (mt gene) Cq – *RNase P* (nuclear gene) Cq.

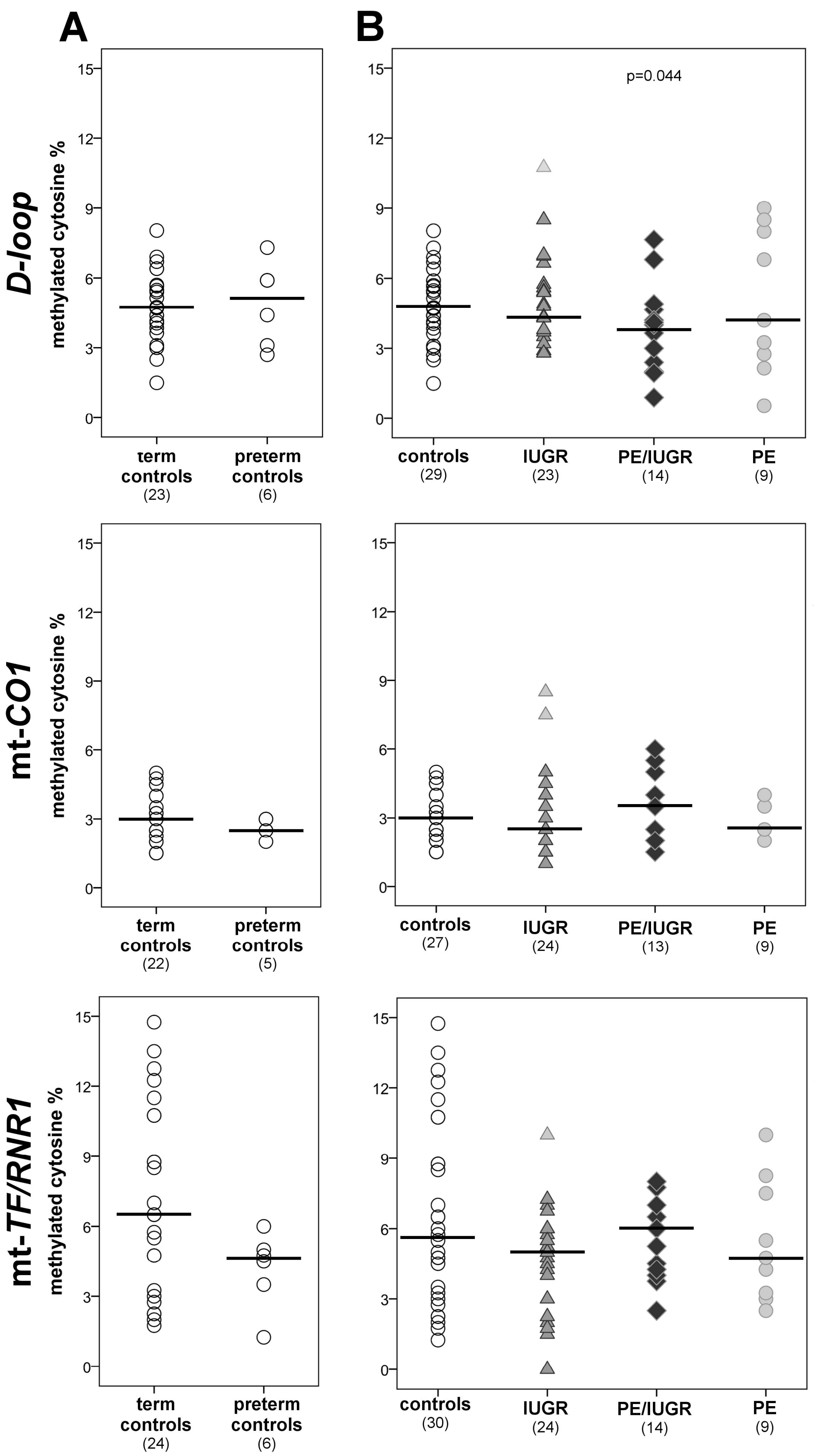
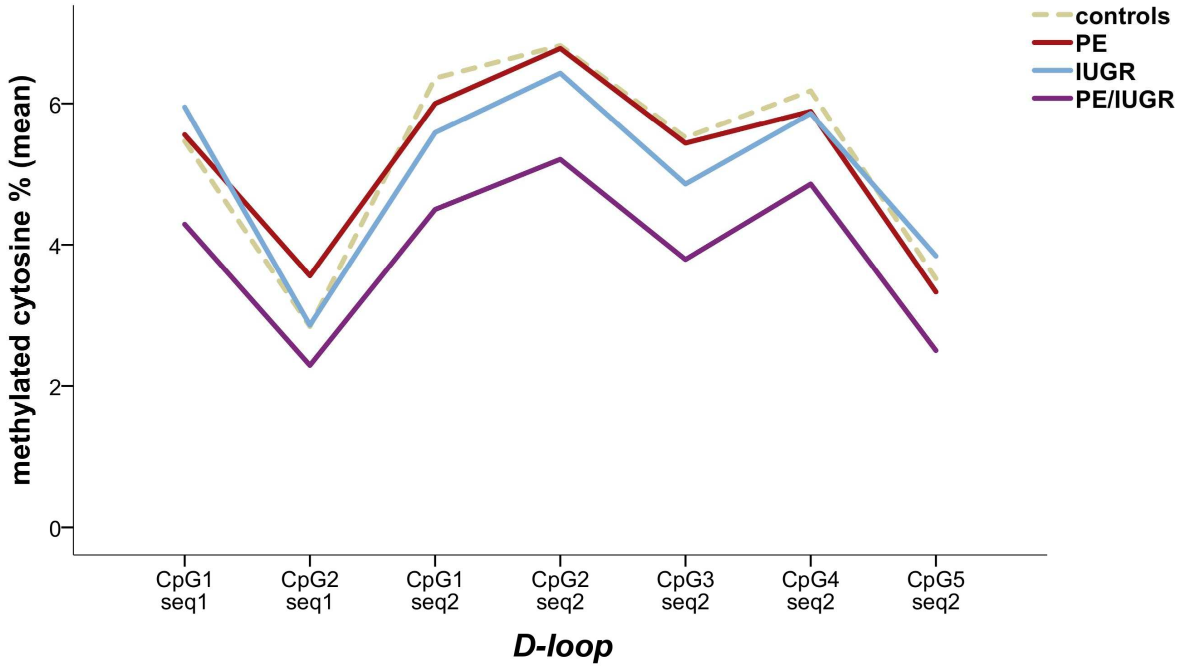


Figure 3: Methylated cytosine percentages in *D-loop*, *CO1*, and *TF/RNR1* mitochondrial regions. Cord blood samples from (**A**) term and preterm controls, (**B**) control (term+preterm), IUGR, PE/IUGR and PE pregnancies. Medians and significant p values *versus* controls are displayed.



Individual CpG methylation in *D-loop locus*. Average methylation percentages for each of the seven analyzed *D-loop* CpGs in 29 control, 23 IUGR, 14 PE/IUGR and 9 PE cord blood samples. PE/IUGR cases present lower methylation levels compared to controls in each CpG.

Supplementary Figure 1

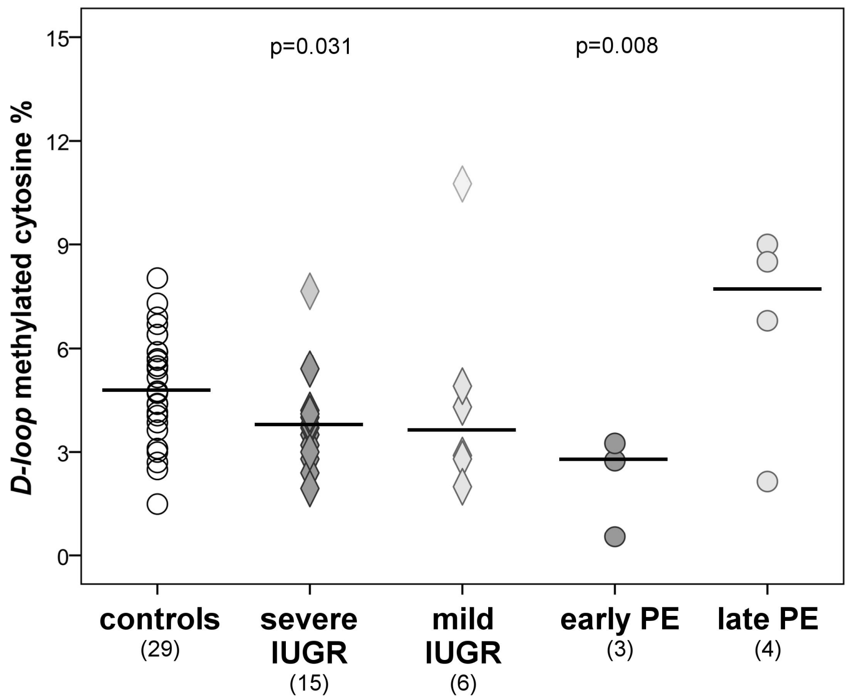


Figure 4: *D-loop* methylated cytosine percentages in pathologic samples divided according to severity. Medians and significant p values *versus* controls are displayed.

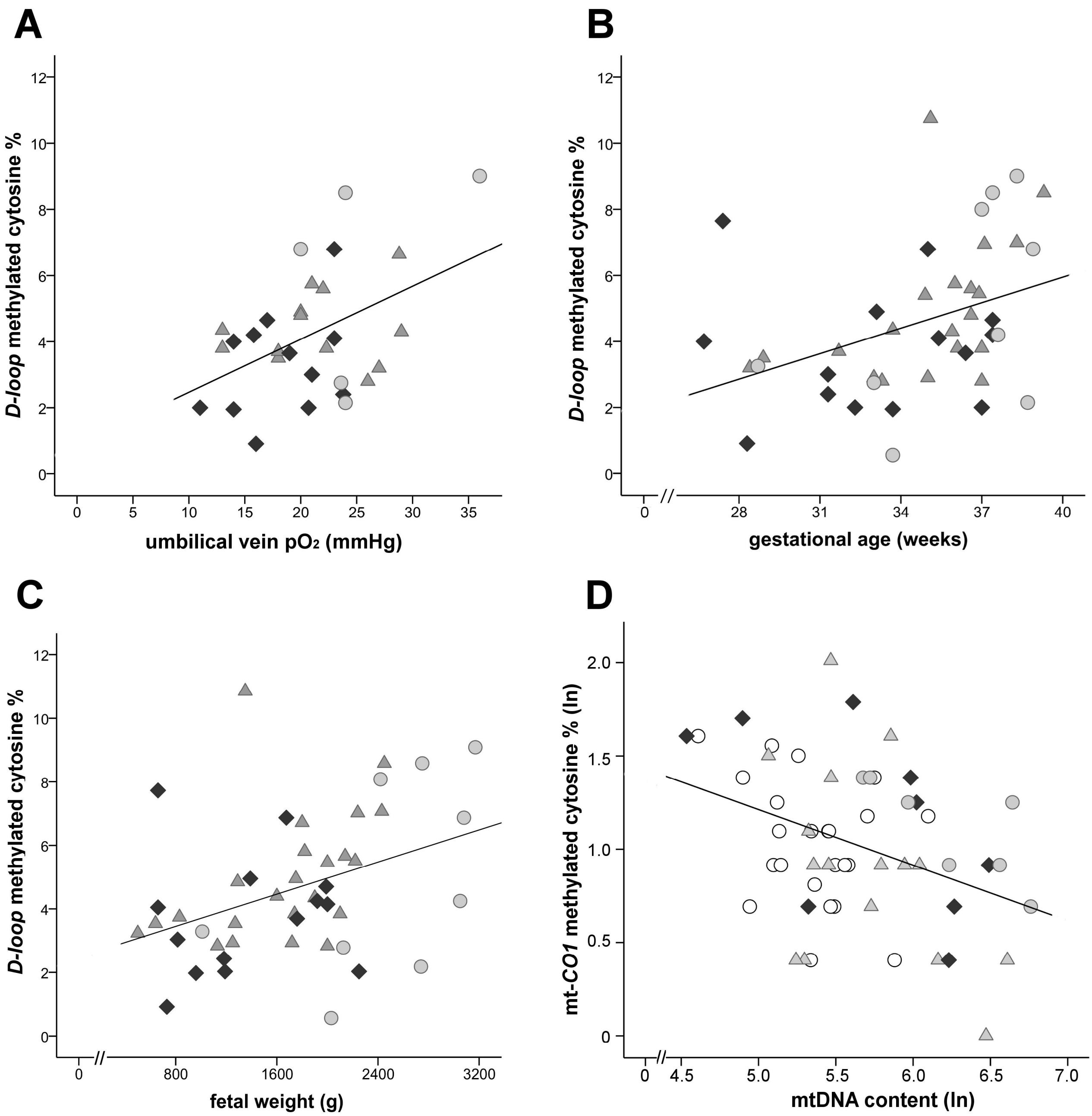
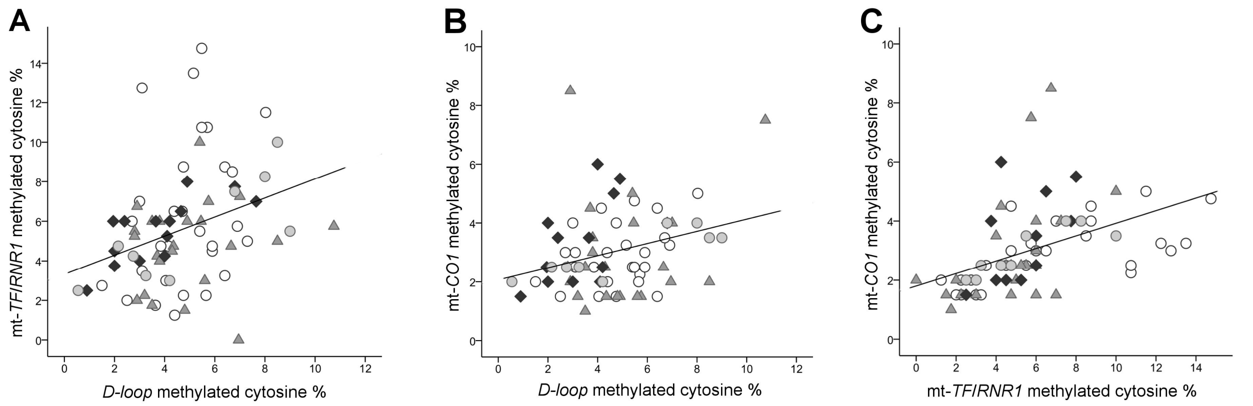


Figure 5: Correlations between molecular and clinical data.

Correlation between *D-loop* methylation and (**A**) umbilical vein oxygen partial pressure (r=0.457, p=0.011), (**B**) gestational age (r=0.378, p=0.013), and (**C**) fetal weight (r=0.385, p=0.008), in pathologic pregnancies. pO_2: oxygen partial pressure; ▲: IUGR, ◆: PE/IUGR, ●: PE.

(**D**): Correlation between mtDNA content and mt-*CO1* methylation (both natural logarithm transformed) in the whole population (r=-0.369, p=0.006). ○ : controls, ▲: IUGR, ◆: PE/IUGR, ●: PE.



Correlations between methylation levels of the three analyzed mitochondrial *loci* in ○ controls, ▲ IUGR, ◆ PE/IUGR and ● PE cord blood samples.
Correlations between **(A)** *D-loop* and *mt-TF/RNR1* (r=0.331, p=0.004), **(B)** *D-loop* and *mt-CO1* (r=0.290, p=0.014),
and **(C)** *mt-TF/RNR1* and *mt-CO1* (r=0.456, p=0.000) methylation levels.

Supplementary Figure 2

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