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Mitochondrial gene expression profiles are associated with intrahepatic cholestasis of pregnancy

Maria Teresa Mella [a], Katherine Kohari [a], Richard Jones [b], Juan Peña [b], Lauren Ferrara [b], Joanne Stone [a, 1], Luca Lambertini [a, b, *, 1]

[a] Department of Obstetrics, Gynecology and Reproductive Science, Icahn School of Medicine at Mount Sinai, New York, NY, USA
[b] Department of Preventive Medicine, Icahn School of Medicine at Mount Sinai, New York, NY, USA

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ABSTRACT

Introduction: Intrahepatic cholestasis of pregnancy (ICP) affects 0.2–2% of pregnant women. While the maternal clinical course of ICP is usually benign, the fetal effects can be severe spanning from spontaneous preterm birth to fetal demise to long term effects on the health of the progeny.
ICP is characterized by high maternal serum levels of bile acids and placental and hepatic bile acids accumulation. Intrahepatic cholestasis, in the non-pregnant state, has been also linked to alterations of the mitochondrial activity attributed to high oxidative stress rates driven by high intracellular bile acids concentrations.
Here we explored the hypothesis that elevated bile acid levels of ICP modify the placental mitochondrial activity.
Methods: By using a set of 12 ICP and 12 control placenta samples, we assessed the expression of all 13 mitochondrial-encoded protein-coding genes and the mitochondrial DNA (mtDNA) relative abundance by real-time PCR. We also assessed the oxidative stress status by measuring DNA damage by ELISA.
Results: We determined that: 1) the expression of *MT-ND4L* ($+53\%$ – $p < 0.01$), *MT-ND4* (-19% $-0.05 < p \leq 0.01$), *MT-ND5* ($+40\%$ – $p < 0.01$), *MT-CYTB* ($+35\%$ – $p < 0.01$) is associated with ICP; 2) the mtDNA relative abundance is not associated with ICP (0.098 in ICP vs 0.118 in controls – $p > 0.05$); 3) the oxidative stress status is associated with ICP (4403.9 pM 8-oxo-dG/μg DNA in ICP vs 3809.8 pM 8-oxo-dG/μg DNA in controls – $p < 0.01$).
Discussion: This preliminary study suggests that mitochondria in placenta respond to high oxidative stress to modify their gene expression which may play an important role in the pathophysiology of ICP.

1. Introduction

Intrahepatic cholestasis of pregnancy (ICP) affects 0.2–2% of pregnant women in their third trimester of pregnancy (80% diagnosed after 30 weeks of gestation) [1,2]. ICP is characterized by high maternal serum bile acids [3] and impaired bile acid pool recirculation [4]. While the maternal clinical course of ICP is usually benign and self-limited, the fetal effects can be severe including high risk of spontaneous preterm birth, meconium stained amniotic fluid, non-reassuring fetal testing, and sudden fetal death [2,3,5]. Importantly ICP also exerts long term effects on the progeny including metabolic syndrome [6] and, in the female, increased risk of ICP [7]. The genetic background of ICP explains some 20% of the disease inheritance [8,9], while the knowledge on the mechanistic basis of ICP is greatly limited.

In the non-pregnant state, hepatic cholestasis is classified as extra- or intrahepatic as determined by the nature of the bile acid pool recirculation impairment. Alterations of the bile acid pool recirculation due to the obstruction of the biliary tree are classified as extrahepatic cholestasis, while those caused by impairments in the bile acids liver reuptake are defined as intrahepatic cholestasis [10]. The temporary alteration of the bile acid pool recirculation caused by impairments in the bile acids reuptake in both placenta [7,11] and maternal liver [11,12] in pregnant women is diagnosed as ICP [4].

In pregnancy the placenta synthesizes active bile acids

* Corresponding author. Department of Preventive Medicine, One Gustave L. Levi Place, Box 1057, New York, NY, 10029, USA.
E-mail address: luca.lambertini@mssm.edu (L. Lambertini).
[1] These authors contributed equally to this work.

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transporters [13–16] under hormonal control [17,18]. The placenta, together with the maternal liver, forms a "placenta-maternal liver tandem" that both supports the yet immature fetal bile acid pool recirculation [19] and protects the fetus from the accumulation of harmful cytotoxic bile acids in the fetal blood. Failure of this system leads to high intracellular bile acid levels that increase the cellular and mitochondrial oxidative stress (OS) affecting mitochondrial respiration and morphology [20–23].

Importantly, mitochondria are really sensitive to high OS rates as they are already routinely involved in controlling the OS generated by the electron transport chain (ETC) of the oxidative phosphorylation (OXPHOS) that they host. The OXPHOS produces over 80% of the ATP needed to carry out housekeeping and specialized cellular functions earning to mitochondria the status of the "powerhouse of the cell" [24]. High mitochondrial OS rates are known to reduce the OXPHOS efficiency mostly affecting those vital organs with high metabolic rates and peculiar homeostatic requirements like, in pregnancy, the placenta. Placental oxygen consumption is in fact second only to that of the fetal brain [25,26] while the placenta also mainly uses glucose to support its high pace metabolism [27,28]. Such metabolic setup in the placenta is required to support and drive embryonic development by coordinating the different phases of embryogenesis and serving as the interface for maternal-fetal interactions [29].

In this pilot investigation we set out to explore the role of the mitochondrial activity in ICP by studying the association between ICP and the placental expression of all 13 mitochondrial-encoded protein-coding genes, the mitochondrial DNA (mtDNA) content and the cellular OS rates in a set of 12 ICP and 12 control placenta samples.

2. Materials and methods

2.1. Study population

This study was approved by the Institutional Review Board at Icahn School of Medicine at Mount Sinai. ICP subjects were identified if they had total maternal serum bile acids $\geq$7 μmol/l. Control subjects were identified from the Mount Sinai Pregnancy Biobank collection. The exclusion criteria included, for both groups, maternal age <18 years or $\geq$35 years, multiple gestations, underlying maternal liver disease, preeclampsia, fetal growth restriction and fetal anomalies. The additional exclusion criteria was gestational age at delivery <34 weeks for ICP and <37 weeks for controls.

2.2. Placenta tissue collection

All placentas were sampled by excising one full-thickness biopsy from each of the 4 placenta quadrants midway from the cord insertion and the placental rim, within 2 h from the delivery. Biopsies were processed by removing the maternal and fetal membranes and washing them in sterile phosphate buffered saline. Biopsies were then blotted dry, snap-frozen in liquid nitrogen and stored at −80 °C.

2.3. DNA and RNA extraction

Placenta biopsies were sampled by using the CXT 350 frozen sample aliquotter (CryoXtract – Woburn, MA, USA) that allows for coring frozen biopsies to retrieve tissue aliquots for downstream applications without thawing the original biopsies. Tissue aliquots where then homogenized into processing tubes with metal beads by shaking at 30 Hz for 3 min with the TissueLyser (Qiagen – Valencia, CA, USA). DNA and RNA were extracted with the Maxwell 16 automated DNA/RNA extraction equipment (Promega – Madison, WI, USA) and quantified with Nanodrop spectrophotometer (Thermo Electron North America – Madison, WI, USA).

Total RNA aliquots were converted into cDNA using the iScript cDNA Synthesis Kit (BioRad - Hercules, CA, USA) following manufacturer's instructions. iScript cDNA Synthesis Kit uses a random hexamer primer to reliably amplify all RNA transcripts.

2.4. Mitochondrial gene expression

We tested the expression of all 13 protein-coding mitochondrial-encoded genes by using primer sets previously designed and validated [30]. Primers all properly work at the annealing temperature of 63 °C thus allowing for thorough randomization of both samples and genes in each real-time PCR plate.

Gene expression was measured by standard real-time PCR in Roche 480 light cycler (Roche Diagnostics – Indianapolis, IN, USA). All reactions were run in triplicate and repeated if the standard deviation between the triplicates was >1 cycle. Expression values were normalized by first calculating the medians for the expression values of the 13 genes tested per each subject. The median of the subject medians was then calculated and the correction value was determined by subtracting the subject median to median of subject medians. The subject-specific correction value was then applied to each gene.

The expression of the housekeeping gene *ACTB* was used to validate the mitochondrial expression data. The lack of mitochondrial housekeeping genes together with the completely different nature of the transcription process between the nuclear DNA (nDNA) and mtDNA warranted the use of a nuclear validated housekeeping gene to verify that the expression variation observed for the mitochondrial-encoded genes was not due to real-time PCR inter-plate variability.

2.5. Mitochondrial DNA relative abundance analysis

mtDNA relative abundance was measured by a quantitative real time PCR assay that determines the corrected difference between the Ct values originated by a primer set that uniquely bind the nDNA at the human β globin gene (*HBG*) and a primer set that uniquely bind the mtDNA at the 16S rRNA, as previously described [31].

The correction was obtained by generating a calibration curve from a series of 5 dilutions of a reference total DNA pool made out of 50 ng of total DNA from each of the 12 control samples included in this study. The DNA serial dilution decrementally ranged from 25 ng/μl to 1.56 ng/μl. The median of the difference between nDNA and mtDNA Ct values across the serial dilutions of the DNA pool was used to adjust the same differences calculated for each sample individually.

The sample-specific mtDNA relative abundance was finally expressed as:

$$\text{mtDNA Relative Abundance} = \text{Sample}\,(Ct_{nDNA} - Ct_{mtDNA}) - \text{Std Median}\,(Ct_{nDNA} - Ct_{mtDNA})$$

where: Sample $(Ct_{nDNA} - Ct_{mtDNA})$ = sample specific difference between nDNA and mtDNA Ct values; Std Median $(Ct_{nDNA} - Ct_{mtDNA})$: = median of the differences between nDNA and mtDNA Ct values from the total DNA calibration curve.

2.6. Oxidative stress measurement

We determined the OS status by using the 8-oxo-dG ELISA II kit (Trevigen – Gaithersburg, MD, USA) following manufacturer's instructions. This kit uses 8-hydroxy-2′-deoxyguanosine (8-oxo-dG)