La sete di conoscenza mi spinge
ad andare più lontano di quanto
nessuno sia mai stato prima di me.
Mi porta al di la' dei limiti del possibile..."
James Cook

UNIVERSITÀ DEGLI STUDI DI MILANO

Facoltà di Medicina e Chirurgia

SCUOLA DI DOTTORATO di

Scienze Fisiopatologiche, Neuropsicobiologiche e Assistenziali del Ciclo della Vita

DIPARTIMENTO di *Scienze Biomediche e Cliniche 'Luigi Sacco'*

CORSO DI DOTTORATO in

Scienze Assistenziali del Ciclo della Vita- Ciclo XXVII

Ex vivo and *in vitro* models to study the effects of hypoxia and inflammation on human placental mitochondria

Settore scientifico-disciplinare MED 40

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ANNO ACCADEMICO 2013-2014

1 Abstract

Intrauterine Growth Restriction (IUGR) is a pregnancy-related pathology characterized by a placental insufficiency phenotype and a multifactorial etiology that still needs to be completely clarified. IUGR is associated with increased risk of maternal and neonatal perinatal mortality and morbidity and a tendency to develop cardiovascular and metabolic pathologies in the adulthood. A deeper knowledge of the alterations occurring in IUGR has therefore become essential to find therapeutic tools to prevent fetal, neonatal and future adult complications.
A specific placental phenotype has been associated with IUGR, characterized by placentation defects, altered transport of oxygen and nutrients to the fetus, impaired mitochondria content and increased oxidative stress (OxS).
Mitochondria (mt) are eukaryotic ubiquitous organelles whose number range from hundreds to thousands of copies per cell. As they are the fuel stations of all cells, more than 95% of ATP is synthesized in these organelles Besides this well-known function, many essential pathways involve mitochondria, such as mt biogenesis. Mt biogenesis is a complex of mechanisms needed to mitochondria *ex-novo* creation: mt DNA duplication and translation of mt factors controlling the transcription machinery that produce all respiratory chain complexes (RCC). IUGR hypoxic features, and the consequent higher OxS, affect mitochondria as showed by *in vivo* models increased mt oxygen consumption trigger by hypoxia or *in vitro* downregulation of mt biogenesis.

The aim of this study was to investigate, by *ex vivo* experiments and *in vitro* models, different types of placental cells to deeper characterize the placental insufficiency features of IUGR, with specific attention to the consequences of its hypoxic environment.

IUGR and physiological placenta bioenergetics were first examined, by analyzing both mitochondrial (mt) content and function in whole placental tissue and in several placental cell types (cytotrophoblast and mesenchymal stromal cells).
Mt DNA content resulted higher in IUGR placentas compared to controls, as well as *NRF1* (biogenesis activator) mRNA levels. Oppositely, both mtDNA and *NRF1* expression levels were significantly lower in cytotrophoblast cells isolated from IUGR placentas compared to controls. The observed divergence between placental tissue and cytotrophoblast cells may suggest that other placental cell types (e.g. syncytiotrophoblast, endothelial cells and mesenchymal stromal cells), that are subjected to different oxygen - and consequently oxidative stress - levels may be responsible for the mt content increase in the whole placental tissue. Moreover, a different exposure to progesterone may also explain this mt content divergence, since progesterone, regulating mt biogenesis, is produced by syncytio but not in cytotrophoblast cells.

In IUGR cytotrophoblast cells, respiratory chain complexes (RCC) showed lower, though not significantly, gene expression levels and no differences in their protein expression compared to controls. In contrast, mt bioenergetics - represented by cellular O_2 consumption - was higher in IUGR *versus* controls, especially in more severe IUGR cases. Thus, despite the protein content of RCC was not altered, their activity was significantly increased in IUGR cytotrophoblast cells, possibly due to a more efficient RCC assembly. Finally, as O_2 consumption resulted inversely correlated to mtDNA in cytotrophoblast cells, a functional (respiration) compensatory effect to the decreased mitochondrial content might be hypothesized.

Estrogen-Related Receptor (*ERRγ*) is a very interesting transcriptional factor involved both in mt biogenesis and function and in estradiol production (through *CYP19* aromatase up-regulation). *ERRγ* and *CYP19* mRNA levels were therefore analyzed, for the first time in human IUGR placentas.

In whole placental tissue *CYP19* showed higher expression in IUGR compared to controls, progressively increasing with IUGR severity. Higher *ERRγ* expression in IUGR cases was also found, though not significantly. These data are consistent with mtDNA and *NRF1* results, thus confirming altered mt biogenesis and content in IUGR and strengthening the hypothesis of a restore attempt made through the stimulation of mt biogenesis. An additional effect of *ERRγ* increase is *CYP19* upregulation. The observed higher *CYP19* expression may indicate a protective mechanism exerted through estradiol against oxidative stress.

Opposite to their placental tissue expression, *ERRγ* levels in cytotrophoblast cells significantly decreased in the IUGR group compared to controls. This is consistent with literature evidences of O_2-dependent *ERRγ* gene expression in trophoblast cells. As well as for mt DNA and *NRF1* levels, other cell types could be responsible for *ERRγ* increase in the whole placental tissue. *CYP19* expression was not significantly different between IUGR and controls in cytotrophoblast cells, though it positively correlates with *ERRγ* levels, but low *CYP19* levels are reported for cytotrophoblast cells, and this might complicate the detection of any difference. Interestingly, a significant positive correlation linked maternal BMI and expression of both *ERRγ* and *CYP19* genes (in whole placental tissue: positive trend/cytotrophoblast cells: negative trend). An estradiol-dependent regulation of leptin production through ER (Estrogen Receptor) – ERR is known. Leptin, an anti-obesity hormone produced also by placenta, increase during. The future measure of plasmatic levels of both leptin and 17β estradiol in maternal blood will verify this speculation.

Then *in vitro* experiments were performed to assess possible biomolecular mechanisms regulating mithocondrial content in Intrauterine Growth Restriction, by culturing primary placental cells under normal oxygen conditions and hypoxia, a typical feature of IUGR. Fluctuations in placental oxygen concentration may generate oxidative stress (OxS), that is enhanced in Intrauterine Growth Restriction condition. As mitochondria are the major producers of intracellular reactive oxygen (O_2) species through free radicals generated by the mt oxidative phosphorylation, altered intrauterine O_2 conditions might affect mt DNA content and function, leading to increased oxidative stress in IUGR placental cells. Using trophoblast primary cell lines could help to understand O_2 conditions that placentas may be exposed to in IUGR pregnancy. Exposure of trophoblast cultures to hypoxia is *an in vitro* model commonly used in the last few years. Preliminary data from performed experiments show that the oxygen lack in cytotrophoblast cells leads to increased mt DNA levels. The evidence that O_2 levels may regulate mt biogenesis in cytotrophoblast cells highlights their deep sensitivity to O_2 conditions. However, further data are needed to confirm these preliminary results, also considering the implied difficulties in adapting the primary cytotrophoblast cultures, very sensitive to O_2 concentration, to an *in vitro* model. A future goal will be reproduce particularly hypoxia/re-oxygenation intervals characterizing placental insufficiency and generating OxS and measuring cell apoptosis levels and autophagy markers (e.g. TNF-α, p53, caspases).

Finally, *in vitro* experiments were performed to isolate and characterized p-MSCs from physiological and affected by IUGR placentas. p-MSCs have never been investigated before in IUGR pregnancies, but their role have been recently studied in preeclamptic placentas. PE p-MSCs show pro-inflammatory and anti-angiogenic features, that may result in abnormal placental development. In the performed p-MSCs cultures, mesenchymal markers enrichment and multipotent differentiation abilities confirm the successful isolation and selection of a mesenchymal stromal cell from placental membranes and basal disc of both physiological and IUGR placentas. As attested by flow cytometry data, the p-MSC population is earlier selected in IUGR placentas: this faster selection might represent a compensatory mechanism to metabolic alterations occurring in IUGR placental cells and/or to the adverse IUGR placental environment. During placenta development, the lower proliferation rate characterizing IUGR pMSCs could impair the primary villi formation and consequently trophoblast development, since MSCs both serve as structural of trophoblast cells.

Moreover, IUGR p-MSCs population display lower endothelial and higher adipogenic differentiation potentials compared to controls. During pregnancy, pMSCs usually contribute to both vasculogenesis and angiogenesis Interestingly, several studies report some alterations

in maternal and fetal endothelial progenitor or in the angiogenic capacity of IUGR placental cells. Opposite to endothelial differentiation ability, the adipogenic potential in pMSCs from IUGR is increased compared to controls: as these changes are evident early in life, the predisposition to obesity may be programmed in utero. To further characterize IUGR pMSCs, their mitochondrial (mt) content was investigated by measuring *NRF1* and Respiratory Chain *UQCRC1* and *COX4I1* gene expression levels. Mesenchymal stem cell metabolism is known to be mainly anaerobic, with a shift towards an aerobic mitochondrial metabolism reported during differentiation. Interestingly, p-MSCs cultured with no differentiating medium present a trend towards higher *NRF1*, *UQCRC1* and C*OX4I1* expression levels in IUGR basal disc samples compared to controls and higher *COX4I1* levels in IUGR placental membranes; these differences are not statistically significant likely because of the low sample number. Nevertheless, they might account for metabolic alterations in IUGR p-MSCs, showing a possible shift to aerobic metabolism, with the loss of the metabolic characteristics that are typical of multipotent and undifferentiated cells.

The different gestational age between cases and controls, typical of all IUGR *versus* term-placentas studies, is a possible limit that associate all the performed experiments. However, any significant correlation between gestational age (ge) and the O2 consumption of CIV (which presents the highest significance between IUGR and controls), ge and mt DNA levels, ge and ERRγ/CYP19 expression, ge and p-MSCs. CYP19 gene expression have been analyzed assuming that it may represent an index of aromatase content in placental tissue. However, post-translational modifications (glycosylation and phosphorylation) may occur, affecting its functional activity. Finally, a potential limitation of placental mesenchymal stromal cells is that the analysis was performed on IUGR placentas at delivery, whereas placental abnormal development of IUGR pathology is supposed to start already at the beginning of placentation.

Taken together, reported data highlight mitochondrial alterations occurring in placentas of Intrauterine Growth Restricted pregnancies, through *ex vivo* and *in vitro* approaches.
These results shed genuine new data into the complex physiology of placental oxygenation in IUGR fetuses. Mitochondrial content is higher in IUGR total placental tissue compared with normal pregnancies at term. This difference is reversed in cytotrophoblast cells of IUGR fetuses, which instead present higher mitochondrial functionality. These findings suggest different mitochondrial features depending on the placental cell lineage.
Indeed, our results on placental Mesenchymal Stromal Cells, showed higher levels of genes accounting for mitohcondrial content and function. The increased placental O2 consumption

by placental tissue may represent a limiting step in fetal growth restriction, preventing adequate O_2 delivery to the fetus. This limitation has potential consequences on fetal O_2 consumption both in animal models and in human IUGR.

2 Introduction

2.1 The PLACENTA

2.1.1 Morphology and Function

The placenta, the primary interface between the fetus and the mother, plays an important role in fetal development and growth.

At the end of pregnancy, the placenta presents a discoid shape and average weight between 500 and 600 g.

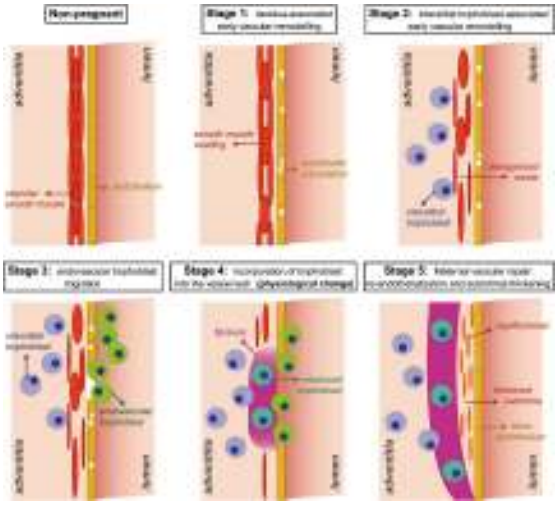
The human placenta is hemocorial and presents two distinct surfaces: a fetal side, oriented to the fetus, and a maternal one connected to the uterine wall.

Examinating the human placenta with a cross-sectional glance from the mother side to the fetus, we can observe:

- ✓ the basal decidua with its maternal origin, made by uterine mucosa
- ✓ the chorionic villi, originated by the chorionic layer
- ✓ the chorion, from which the villi network originates. The outer layer is made by syncytiotrophoblast, while the inner part is made by cytotrophoblast cells. The inter-villous space, covered by a trophoblast layer, includes the villi and the maternal blood circulation
- ✓ the amnios, which covers the fetal side of the placenta, envelopes the cord until its ombelical insertion. The fetal cord includes one vein (blood enriched with oxygen and nutrients) and two arteries (blood enriched with waste substrates flowing from the fetus to the mother).

The main functions of the placenta [Jansson and Powell, 2007] include:

1) modulating maternal immune response to prevent immunological rejection of the conceptus
2) facilitating the exchange of respiratory gases, water, ions, nutrients and wastes between the maternal and fetal circulations
3) producing and secreting hormones, cytokines and signalling molecules required to pregnancy maintenance and to ensure placental/fetal development.



Maternal blood supply to the placenta is established at the end of the first trimester of human pregnancy, with maternal vessels entering the placental intervillous space from the transformed spiral arterioles [Jones *et al.*, 2007; Marconi and Paolini, 2008]. In details, extravillous trophoblast cells colonize the maternal decidua (**figure 2.1**) and subsequent remodelling of the spiral arteries leads to the formation of heavily dilated conduits that lack maternal vasomotor control [Pijnenborg *et al.*, 1980].

Figure 2.1: Diagram showing the different steps in uterine spiral artery remodelling, starting from the non-pregnant condition. Stage1: the earliest stage in vascular remodelling. Stage2: invasion of stromal and perivascular tissues by trophoblast, associated with further disorganization of the vascular smooth muscle layer. Stage3: only then, endovascular trophoblast appears. Stage 4: trophoblast becomes embedded intramurally within a fibrinoid layer, which replaces the original vascular smooth muscle. Stage5: finally re-endothelialization occurs, accompanied by the appearance of subintimal cushions with a-actin immunopositive myointimal cells.

This progressive reduction of vessel resistances allows the creation of a low-pressure circulation needed for a functional placenta. The maternal vessels transformation, which continues until the end of the second trimester of pregnancy, is indeed believed to prevent spontaneous arterial constriction and intermittent perfusion, facilitating the delivery of a constant supply of blood to the materno-fetal interface at an optimal pressure and velocity. An inadequate or incomplete remodeling of the spiral arteries, characterised by shallow trophoblast invasion and narrow-bore arteries retaining muscular walls, causes fluctuations of the uterus-placenta blood flow. This may cause hypoxia-reperfusion injury and reduced nutrient exchange [Harris, 2009] and has been associated with preeclampsia [Hutchinson *et al.*, 2009; Dekker *et al.*, 1998], pre-term birth [Kim *et al.*, 2003] and some cases of fetal growth restriction (FGR) [Khong *et al.*, 1986] or intrauterine growth restriction (IUGR) [Mayhew *et al.*, 2004].

The barrier between the maternal and the fetal circulations consists of three fetal cellular layers (**figure 2.2**) [Sibley *et al.*, 2010]:

(i) the syncytiotrophoblast, a multinucleated epithelial layer formed after the fusion of the mononucleated villous cytotrophoblasts. Syncytiotrophoblast cells represent the main

regulator of substrate exchange, bearing nutrient transporters on both plasma membranes [Lager and Powell, 2012]

(i) villous stromal tissue, made by the mesenchymal cells, mesenchymal-derived macrophages (Hofbauer cells) and fibroblasts. Hofbauer cells synthesize VEGF and other proangiogenic factors that initiate placental vasculogenesis

(ii) the fetal capillary endothelium with its fetal vascular cells that include vascular smooth muscle cells, perivascular cells (pericytes) and endothelial cells [Wang and Zhao, 2010].

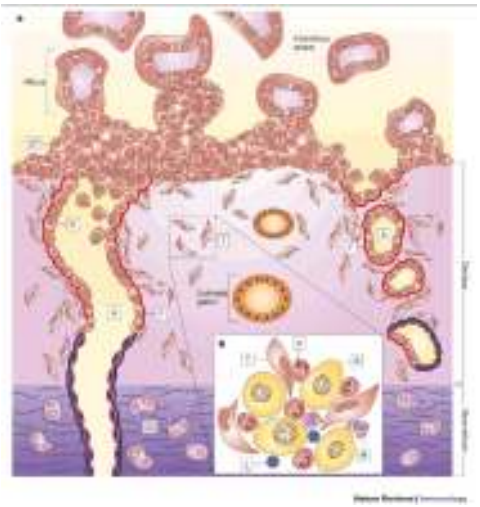


Figure 2.2: Trophoblast population in the maternal-fetal interface during the 1st trimester (from Moffett-King, 2002)

Trophoblast Cells

From very early in development, the mammalian embryo signals its presence to the mother in order to establish the pregnancy via a specialized cell type, the trophoblast [John and Hemberger, 2012]. Trophoblasts are placental cells that play an important role in embryo implantation and interaction with the decidualised maternal uterus. The villi, known as the placental nutrient-transporter unit, are made by trophoblast, mesenchymal cells and placental blood vessels. Two layers of trophoblasts surround the villi: a single layer of mononuclear cytotrophoblasts that fuse together to form the overlying multinucleated syncytiotrophoblast layer, which covers the entire surface of the placenta. The syncytiotrophoblast cells keep direct contact with the maternal blood that reaches the placental surface, thus facilitating the exchange of substrates. In addition, cytotrophoblasts in the tips of villi can differentiate into another type of trophoblast called the extravillous trophoblast (EVT). EVTs grow out from the placenta and penetrate into the decidualised uterus, during the colonization process described before. Although they never contribute directly to the body of the embryo itself, these trophoblast cells are critical for implantation, interacting locally with the maternal uterine environment to ensure sufficient blood supply towards the implantation site and to prevent immune rejection of the semi-allogeneic fetus [Rossant and Cross, 2001]. As pregnancy

proceeds, trophoblast cells develop specialized functions to enable the efficient transport of nutrients to the fetus and the disposal of waste products from it.

Mesenchymal Stromal Cells

Mesenchymal stromal cells (MSCs) are the predominant cellular component in placenta and serve as structural support for trophoblast villi and vascular network [Nuzzo *et al.*, 2014; Klein, *et al.*, 2011; Demir *et al.*, 1989]

MSCs were first discovered in 1968 by Friedenstein and colleagues as adherent fibroblast-like cells in the bone marrow capable of differentiating into bone. Nowadays mesenchymal stromal cells have been isolated and characterized from several adult and fetal tissues including adipose tissue, dermis, umbilical cord blood, placenta and amniotic fluid. In an effort to better characterize MSCs, the International Society for Cellular Therapy defined the following criteria: MSCs must be adherent to plastic under standard tissue culture conditions, express certain cell surface markers such as CD73, CD90, and CD105, and lack expression of other markers including CD45, CD34, CD14, CD11b, CD79α, or CD19 and HLA-DR.

Interesting properties of MSCs are the following:

- ✓ Homing Efficiency: MSCs have a tendency to home to damaged tissue sites
- ✓ Differentiation Potential: as typical multipotent cells, MSCs have shown the capability to differentiate into a variety of cell types, including adipocytes, osteoblasts, chondrocytes, myoblasts, and neuron-like cells
- ✓ Production of Trophic Factors: evidences show that MSCs could act as a trophic factor pool secreting growth factors and other chemokines which induce cell proliferation, angiogenesis and prevent cell apoptosis
- ✓ Anti-inflammatory and immunomodulatory properties: MSCs can suppress the excessive responses from T cells, B cells, dendritic cells, macrophages and natural killer cells.

Only few studies have focused on human placental MSCs (p-MSCs) in placental insufficiency pathologies, and all of them have addressed preeclampsia (PE) [Nuzzo *et al.*, 2014; Rolfo *et al.,* 2013; Chen *et al.,* 2013; Wang *et al.,* 2012; Hwang *et al.,* 2010; Portmann-Lanz *et al.,* 2010]. Human MSCs isolated from PE placentas and deciduas display decreased proliferation and interestingly show pro-inflammatory and anti-angiogenic characteristics [Rolfo *et al.,* 2013; Chen *et al.,* 2013; Wang *et al.,* 2012], that may lead to impared trophoblast development and invasive capacity, and defective placental vasculogenesis.

Currently, no data have been reported on p-MSCs in IUGR.

2.1.2 Nutrient transfer to the fetus

A physiological intrauterine fetus growth depends on maternal nutrition, and on efficient supply of nutrients from the mother to the fetus, in other words on the placenta ability to transport substrates from the maternal to the fetal circulation [Marconi and Paolini, 2008]. Indeed, in a physiological intrauterine development, the major macro and micro nutrients exchanges through the maternal-fetal circulation take place inside the placenta, with the two circulations being kept separate by the placenta barrier. The placental barrier is the tissue layer composed by syncytio and cytotrophoblast cells, stromal and endothelial cells. It is a semipermeable layer, in fact it allows the transfer of highly permeable molecules (such as gases, oxygen and carbon dioxide), which is influenced by blood flow and occurs via simple diffusion, whereas less permeable substrates are transferred through passive and active transport processes. The major substrates required for fetal growth include oxygen, glucose, amino acids and fatty acids [Lager and Powell, 2012; Sibley *et al.*, 2010; Jones *et al.*, 2007]. Maternal blood, inside the intervillous space, draws the demanding substrates, while the fetus reaches the maternal circulation through the villi.

The human placenta capacity to provide substrates to the fetus, also referred to as placental efficiency (which can be assessed from the fetal-placental weights ratio = grams of fetus per gram of placenta), relies on multiple factors: placental size and morphology, uterine blood flow, operative substrates carriers and the nutrients production/consumption rates. During intrauterine development all these factors are tightly related to each others and can be modulated by epigenetical, environmental or maternal/fetal changes [Fowden *et al.,* 2008]. Indeed, placental efficiency measurements provide the extent to which placental adaptations during intrauterine development have occurred in order to meet fetal growth demands [Fowden *et al.,* 2008].

Oxygen levels during pregnancy trimesters

Oxygen plays a critical role in fetus development, but also in the development and function of the placenta. Oxygen (O_2) diffuses from the maternal to the fetal circulation across the epithelial layer of the placental barrier [Jauniaux *et al.,* 1994], which itself consumes O_2. This O_2 consumption generates a transepithelial oxygen partial pressure (pO_2) difference whose magnitude depends upon the rate of umbilical and uterine blood flow, fetal and maternal blood oxygen carrying capacity, haemoglobin oxygen binding affinity, placental surface area and placental permeability [Laszo *et al.,* 1990].

The placenta acts uniquely as both a conduit of oxygen to the fetal circulation and a significant consumer of oxygen, taken up from maternal circulation, in order to support its own energy demands. The human placenta needs approximately 80% (in mid-gestation) and 40%–60% (in late gestation) of the total oxygen uptake by the pregnant uterus, of which one third is used for *de novo* synthesis of proteins and another third for cell respiration and metabolism (in particular for the maintenance of the cation gradient across the mitochondrial membrane [Murray, 2012; Carter, 2000]. Moreover, despite decreases in maternal oxygenation and uterine blood flow, the pO_2 gradient across the placenta remains constant in order to sustain fetal oxygen delivery at a normal rate [44]. The level of placental oxygen differs depending on each gestational stage and on the ranges of normoxia, which is defined as the oxygen concentration needed for an adequate supply to the placental–fetal unit (**figure 2.3**). During the first trimester, the conceptus develops in a low oxygen environment (pO_2 at 7-10 gestational weeks: <20 mmHg) until the utero-placental vasculature can provide efficient gas exchange that favors organogenesis in the embryo and both cell proliferation and angiogenesis in the placenta. Later in pregnancy, higher oxygen concentrations are required to support the rapid growth of the fetus. This transition, which appears unique to the human placenta, must be negotiated safely for a successful pregnancy [Burton, 2009].

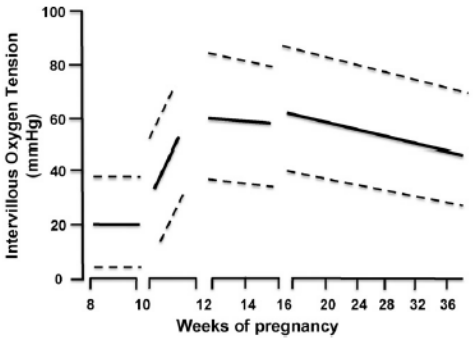


Figure 2.3: The means and 95% confidence intervals of oxygen partial pressure – dashed lines – throughout gestation in the intervillous space in the human. Values are derived from *in vivo* measurements adapted from Zamudio (2011).

2.1.3 Placental endocrine function: estrogens production

One of the most intriguing roles played by placenta is the production of a wide array of hypothalamic or pituitary hormones, which are needed to conserve and later mobilize maternal nutrients. Most of them are protein hormones, while estrogen and progesterone are steroid ones.

The human placenta, specifically its trophoblast cells, produces a huge quantity of steroid and protein hormones, which is greater in amount and diversity than that of any single endocrine tissue in all mammalian physiology [Williams-Obstetrics]. Placental hormones display endocrine and paracrine functions: estrogens stimulate uterine blood flow, influence progesterone production and steroid metabolism, as well as influence maternal weight gain and prepare breasts for lactation.

During pregnancy, the placenta produces a huge amounts of estrogens using blood steroidal precursors from the maternal and fetal adrenal glands. A normal pregnancy presents a hyperestrogenic state at term: there is a 10-to-20-fold increased metabolic clearance rate of plasma estrogens in pregnant (estradiol pg/ml= <20-443) compared to non-pregnant women (estradiol pg/ml= 6137-3460) [Gant, 1971].

Although this estrogen production is mainly due to the placenta, in trophoblast cells neither cholesterol nor progesterone can serve as precursor for estrogen biosynthesis, so that it becomes dependent upon maternal and fetal adrenal production of precursors as dehydroepiandrosterone-sulfate (DHEA-S). The placenta has an exceptionally high capacity to convert C19 steroids, and in particular DHEA-S to estradiol. This conversion needs the expression of four key enzymes principally located in syncytiotrophoblast cells (**figure 2.4**): steroid sulfatase (STS), 3β-hydrosteroid dehydrogenase type 1 (3β-HSD1), and finally Cytochrome P450 (CYP19), which converts androstenedione to estrone, and 17β-hydrosteroid dehydrogenase type 1 (17β-HSD1) that transforms estrone in estradiol [Bonefant, 2000]. The expression of CYP19 is in turn regulated by 17β-estradiol with a reverse action.

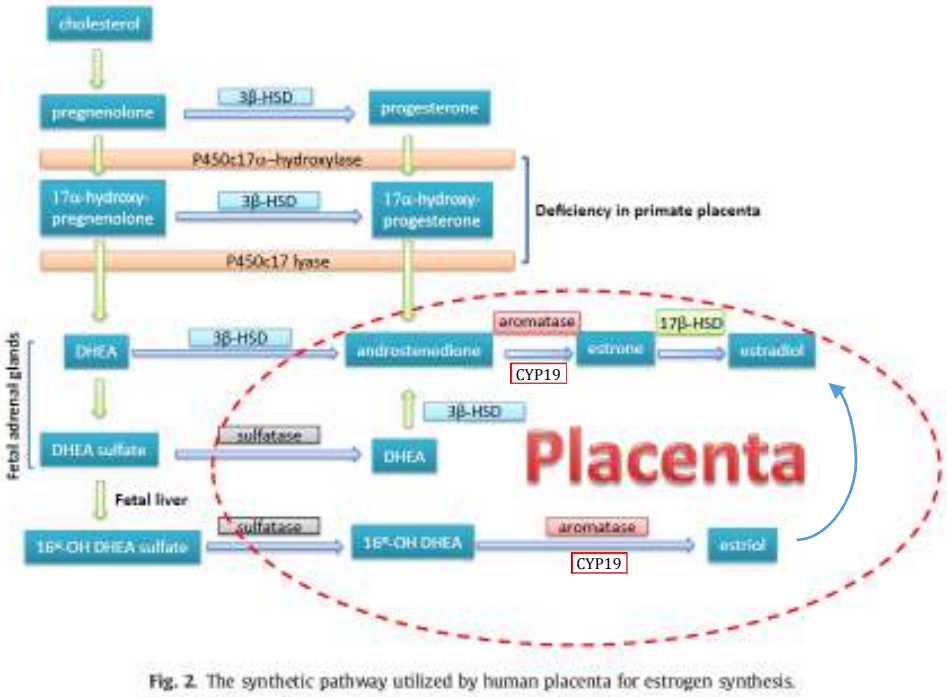


Fig. 2. The synthetic pathway utilized by human placenta for estrogen synthesis.

Figure2.4: The syntetic pathway of estrogen production in human placenta (adapted from Li et al. 2014).

2.2 FETAL GROWTH

Fetal growth is the result of genetic potential modulated by endocrine and nutritional environment [Cetin and Alvino, 2009] and is dependent on substrates supply through their transport regulation. These supply processes hang also on morphological characteristics of the placenta, such as its size and morphology, blood flow and vascularity. Therefore placental nutrient transfer capacity is specifically regulated by signals of fetal, maternal and placental origin in an effort to control fetal growth.

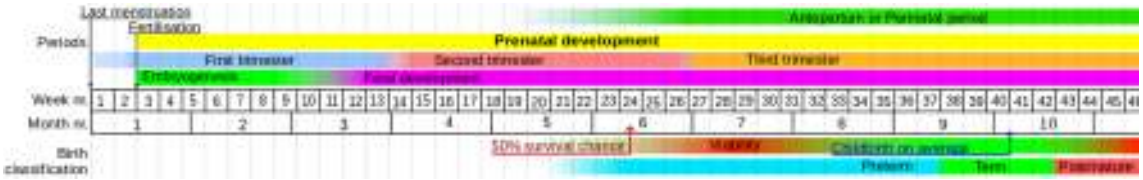


Figure 2.5: stages during pregnancy. Embryogenesis is marked in green. Weeks and months are numbered by gestatation.

The fetal development could conventionally be classified in two main periods of growth: the prenatal or antenatal development, and the perinatal period (**figure 2.5**). Prenatal or antenatal

development is the process in which a human embryo or fetus gestates during pregnancy, from fertilization until birth. After fertilization, the process of embryogenesis (the early stages of prenatal development) begins. By the end of the 10th gestational week (gw) the embryo has acquired its basic form and the next period is required for fetal organs development. The perinatal period (or "antepartum") is "around the time of birth". In developed countries and at facilities where expert neonatal care is available, it is considered from 22 completed weeks of gestation (154 days- when birth weight is normally 500 g) to 7 completed days after birth. In many developing countries, the starting point of this period is considered around the 28th gw (or weight more than 1000 g). Practically, however, antepartum usually refers to the period between the 24th - 26th gw and after birth.

Pregnancy is also divided into trimesters, each lasting about 12-14 weeks. The first trimester occurs from week 1st to the end of week 13th. The second trimester usually ends around the 26th week and consists of the 4th, 5th and 6th completed months. The third trimester can end anywhere between the 38th-42nd week and includes the 7th, 8th and 9th completed months of pregnancy.

Intrauterine growth can be divided in three main periods:

- ✓ Pre-embrional period: from the conception to 2 weeks of gestation
- ✓ Embrional period: until 8-10 weeks of gestation
- ✓ Fetal period: from 8-10 gw until the end of pregnancy.

2.2.1 Fetal Growth and Fetal Programming

Fetal growth is a complex process due to the interaction of both genetic and environmental factors, as well as epigenetic variations in intrauterine compartment. An altered fetal growth is associated to a higher risk to develop perinatal complications, with related consequences also in the adult life. Indeed, defects during intrauterine development predispose the newborn to cardiovascular and metabolic pathologies during both childhood and adult life, driving the occurrence of long-term changes as asserted in the "fetal programming" theory [Barker *et al.,* 2001]. In a previous work by our group [Cetin *et al.*, 2004], very small birth-weights of the babies (due to poor fetal growth and/or preterm delivery) were associated with substantially elevated risks of metabolic syndrome (dislipidemia, insulin resistance, hypertension), type 2 diabetes and cardiovascular disease in adulthood. However, also macrosomial fetuses could easily run into metabolic complications in their future adult life [Luo *et al.*, 2006].

The mechanisms of such "fetal origins" or "programming" of disease phenomenon remain under investigation. Many known or suspected causes or conditions associated with adverse (poor or excessive) fetal growth or preterm birth have been related to oxidative stress.

2.3 Placental Insufficiency Phenotype: IUGR

Placental insufficiency is a serious pathological condition, with a multifactorial etiology due to morphological and functional alterations in the human placenta. Placental insufficiency phenotype can be associated with maternal (e.g. preeclampsia) or fetal (e.g. Intrauterine Growth Restriction) complications.

The American College of Obstetricians and Gynecologists (ACOG) defines IUGR as *"a fetus that fails to reach his potential growth"* [ACOG, 2001]. Intrauterine growth restriction (IUGR) is clinically identified *in utero* from the 20th week of gestation, when longitudinal sonographic measurements demonstrate a reduction in fetal growth [Pardi *et al.*, 1993], with a shift from the reference ultrasound growth curve greater than 40 centiles [Todros *et al.*, 1987]. IUGR is then confirmed at birth by a neonatal weight below the 10th percentile according to standards for birth-weight and gestational age [Parazzini *et al.*, 1995]. The IUGR condition must be distinguished from that of a Small-for-Gestational Age (SGA) fetus, which presents a birth-weight below the 10th percentile (adjusted for sex gender and gestational age) but not an *in utero* reduction of fetal growth, concerning only the newborn features.

IUGR cases can be classified in two groups based on their severity, by considering two clinical parameters: fetal hearth rate (FHR) and umbilical artery Doppler Velocimetry (pulsatility index, PI - Pardi *et al.*, 1993):
IUGR with normal PI, also displaying normal FHR
IUGR with abnormal PI, including both cases with normal FHR and cases with abnormal FHR
This classification reflects the different stages of placental insufficiency and is associated with significant changes in the nutrients transport through the placenta [Pardi *et al.*, 1993].

Intrauterine Growth Restriction is associated with an increased risk of maternal and neonatal mortality and morbidity [WHO, 2005; Franchi *et al.*, 2004], particularly IUGR neonates have a greater risk of hypoxic-ischemic encephalopathy, intraventricular hemorrhage and necrotizing enterocolitis with longer hospital stays and higher health care costs. IUGR has a 7-15% of incidence in general population, and is also responsible for about 10% of perinatal deaths [Baschat *et al.*, 2004; Alexander *et al.*, 2003].

In the developing world, IUGR is likely to be a consequence of poor maternal nutritional status prior to or during pregnancy, also influenced by preexisting risk factors (see below).
In the developed world, instead, IUGR is commonly a consequence of placental insufficiency.

A specific placental phenotype has been associated with IUGR [Cetin and Alvino, 2009], characterized by placentation defects together with reduced and altered transport of oxygen and nutrients [Cetin *et al.*, 2013; Mandò *et al.*, 2013; Mandò *et al.*, 2011; Pardi *et al.*, 1993] and impaired mitochondrial content [Colleoni *et al.*, 2010].

Known causes or risk factors for IUGR are (**figure 2.6**):

- ✓ maternal (metabolic disorders, such as chronic hypertension or pre-gestational diabetes, cardiovascular/renal or autoimmune diseases, genetic defects, or negative habits such as alcohol and drug abuse or smoking)
- ✓ fetal (infections, congenital malformations and chromosomal abnormalities)
- ✓ placental (insufficiency pathologies, hypoxic lesions, genetic mosaicism).

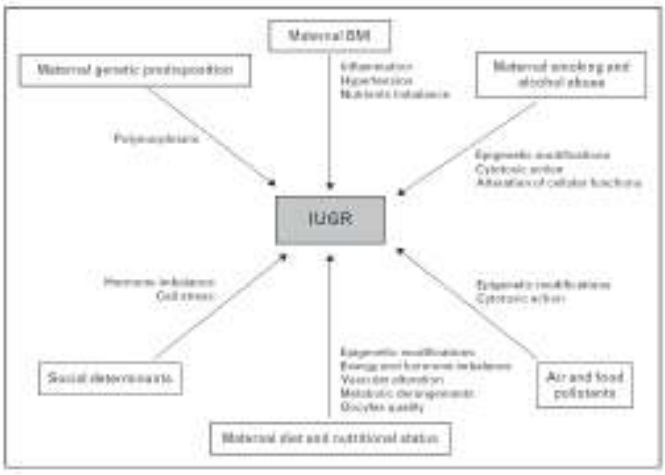


Figure 2.6: Maternal risk factors for IUGR and their mechanisms.

2.3.1 IUGR and Placental Defects

Placental histological defects are usually associated with IUGR as a sign of an inefficient placentation and could be the possible cause of a reduction in substrates supply (nutrients and oxygen) needed for a physiological fetal growth. Many studies show the presence of these morphological abnormalities in both placental villi and blood vessels [Dekker *et al.*, 1998; Salafia *et al.*, 1997]. There are evidences of a strong relationship between fetal and placental weight, more marked in relation to growth restriction severity [Pardi *et al.,* 2002]. Placentas of women with IUGR and/or preeclampsia present evident repeated small hypoxic lesions on their surface (see paragraph 2.4.3), with consequent lipoproteins deposition and thrombotic episodes.

These lesions are often associated with a defective vascular transformation and terminal villous formation, with fewer and thinner villi and vessels - especially in the most severe cases - differently distributed along the placental surface. The pathological placental villi display a 30-50% less invasive capacity than physiological ones [Barut *et al.,* 2010; Dekker *et al.*, 1998]. Moreover, an unbalance between pro- and anti-angiogenic factors is well documented in placentas and maternal blood of IUGR pregnancies, suggesting a role in their pathophysiology [Regnault *et al.,* 2002].

IUGR placenta presents many other failure signs, such as reduced syncytiotrophoblast surface, enhanced placental barrier thickness (composed by trophoblast and endothelial cells), and increased cellular apoptosis [Ishihara *et al.*, 2002]. Beyond abnormal terminal villi, growth restricted placentas show an enhanced number of syncytiotrophoblasts nuclei, whereas a reduction of cytotrophoblasts plus an increase of collagen and laminin deposition in the stromal tissue are found [Macara *et al.*, 1996]. All these evidences are consistent with the ineffective fetal-placental vasculature, characterizing IUGR placentas (**figure 2.7**) [Sibley *et al.,* 2005].

Both placental transport and metabolism are considerably modified in IUGR pregnancies [Cetin *et al.*, 2013; Mandò *et al.*, 2013; Mandò *et al.*, 2011; Pardi *et al.*, 1993]. Usually in growth-restricted placentas weight, surface and permeability changes occur, which together with uterus-fetus blood flow alterations may cause an inefficient perfusion and nutrient supply to the fetus [Pardi *et al.,* 2006]. Generally, all these placental defects lead to a change in the fetal growth path, despite the fetus attempts to adaptation.

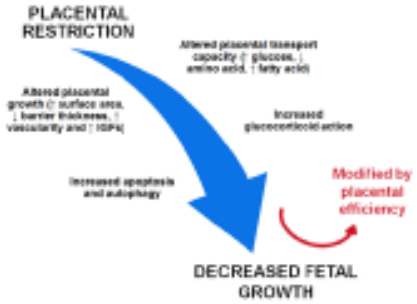


Figure 2.7: Summary of the placental adaptations that occur in the placental insufficiency-induced IUGR fetus and contribute to a decreased fetal growth.

2.4 MITOCHONDRIA

Mitochondria (mt) are ubiquitous organelles present in most eukaryotic cells in variable numbers ranging from hundreds to thousands of copies per cell [Schon, 2000]. Mitochondria are made by a soluble matrix surrounded by a double membrane, an ion impermeable inner membrane and a permeable outer membrane. Early biochemists recognized the importance of mitochondria, as they are the fuel stations of all eukaryotic cells: more than 95% of ATP is synthesized in these organelles through oxidative reactions [Attardi and Schatz, 1988]. Besides this well-known function, there are many essential metabolic pathways involving mitochondria-localized steps, such as nitrogen metabolism, haem biosynthesis, purine and Fe-S clusters synthesis. Moreover, mitochondria play an important role in apoptosis and are involved in signal transduction for cell proliferation [Wallace, 2008]. These important functions demonstrate that mitochondria are essential for maintaining the health of an organism.

2.4.1 Mitochondrial DNA and Biogenesis

Mitochondria and their chloroplast cousins are unique among eukaryotic extranuclear organelles because of their own genetic system. In vertebrates, this system is based on an mt genome consisting of a circular double-stranded DNA (mt DNA). An individual mitochondrion can contain more than one mitochondrial genome: the real number has been estimated to be between 0 and 11 copies with a mean of 2.0 [Scarpulla, 2008]. The amount of mt DNA in a cell could provide a major regulatory point in mitochondrial activity, as the transcription of mitochondrial genes is proportionate to their copy numbers [Malick *et al.*, 2011].

Although mt DNA is quite diverse in the eukaryotic kingdom, the organization of the mammalian mitochondrial genome is significantly conserved [Clayton, 1992b].

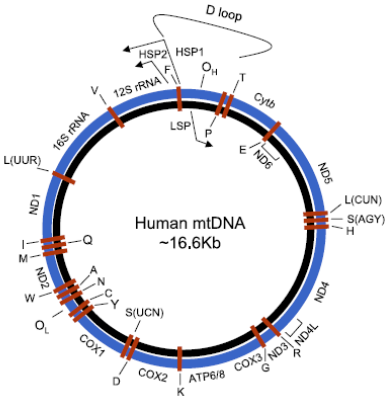


Figure 2.8. Schematic representation of human mitochondrial genome. Genomic organization and structural features of mtDNA in a circular genomic map with heavy (blue) and light (black) strands. Protein-coding and rRNA genes are interspersed with 22 tRNA genes (red bars). The D-loop regulatory region contains the replication origin (OH); arrows showing the direction of transcription. Protein coding genes include cytochrome oxidase (COX), 3-NADHdehydrogenase (ND), ATP synthase (ATPS), cytochrome b (Cyt b).

In particular, the human mitochondrial genome is 16.6-kb-long [Attardi and Schatz, 1988] and its two strands are identified by their varying densities as the heavy (H) or the light (L) strand. Mt DNA

comprises only a small fraction of protein-encoding genes necessary for the molecular architecture and biological functions of the organelle itself. Because of this limited coding capacity, mitochondria are genetically semiautonomous and rely heavily on the expression of nuclear genes. For example, the majority of protein subunits that constitute the five inner-membrane complexes of the electron transport chain and oxidative phosphorylation system are nucleus-encoded (figure 2.8).

In the mitochondrial genome, thirteen genes code for polypeptides that are part of the oxidative phosphorylation system, with twelve of these genes being located on the H strand. Two rRNAs and twenty-two tRNAs are also encoded by the mt genome. The displacement loop (D loop) is a 1.1 kb noncoding control region that is important in replication and transcription. Other mitochondrial proteins are nuclear encoded, then synthesized in the cytoplasm and finally transported to the mitochondria. It is also important to note that mtDNA is not naked, but packaged into a nucleoid structure which serves as a mitochondrial genetic unit [Gilkerson *et al.,* 2008].

Mitochondria are also unique for their Mendelian genetic system. First, mtDNA shows a maternal inheritance pattern (paternal mtDNA is almost never passed on to progeny); second, mitochondria are polyploid, with up to several thousand copies of their genome per cell. Also, replication and transcription are coupled in mitochondria, with a unique machinery that carries out these processes.

Mt biogenesis is defined as the complex of mechanisms needed to the *ex-novo* creation of all the components of new mitochondria: mt DNA duplication, translation of proteins (transcriptional factors) managing the access to the transcription machinery and of the respiratory complexes proteic subunits. Based on extrapolation from the fully sequenced yeast genome, mt biogenesis requires the expression of more than 1000 genes, tightly regulated, which are encoded on both nuclear chromosomes and the mitochondrial genome [Goffart and Wiesner, 2003]. Mt biogenesis control and coordination is managed by the mitochondrial transcriptional factors or activators: a group of single or complexed proteins performing a co-acting action to bind their final target: TFAM (Mitochondrial Transcription Factor A). TFAM exerts its function regulating mt replication and transcription but also mt turnover, by supervising on mt DNA copy number [Campbell *et al.,* 2012]. Among mt biogenesis activators binding TFAM promoter region there are:

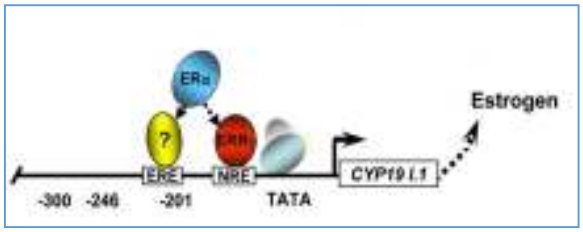
- ✓ NRF1 (Nuclear Respiratory Factor 1): it influences mt biogenesis through its direct bound to TFAM and plays a role in the respiratory chain complexes translation (e.g. Cytochrome C) [Poidatz *et al.,* 2012]
- ✓ PGC1α (PPARγ-coactivator 1α): it is a transcriptional coactivator of PPARγ through its bound with NFR1 and is an enhancer of NRF-1, NRF-2, and TFAM gene expression [Wu *et al.,* 1999]. A higher PGC1α transcriptional activity is associated with an increase of mt DNA content in animal models of cardiovascular pathologies [Bayeva *et al.,* 2013].
- ✓ ERRγ (Estrogen-Related Receptor gamma): it is a nuclear receptor without a known ligand showing a higher homology to Estrogen Receptor (ER) [Giguère *et al.,* 2008]. Interacting with PGC1α e NRF1 it exerts its role in energetic metabolism and cellular differentiation [Ranhotra, 2012].

Assessing Mitochondria Content: mt DNA and Biogenesis Activators

Well accepted biochemical markers to assess mitochondria content in human eukaryotic cells are the mt DNA copy number, the mRNA levels of mt biogenesis transcription factors (e.g. TFAM, NRF1, NRF2 and PGC1α), the expression of mt DNA-encoded proteins and the rates of mt translation [Medeiros, 2008]. Interestingly, the amount of mt DNA has been shown to be directly proportional to total TFAM levels [Ekstrand *et al.*, 2004]. There is a widespread interest in accurately quantifying all these markers, particularly mt DNA, in order to discover an attractive and non-invasive biomarker for predictive and diagnostic purpose [Malick et al., 2011].

Estrogen-Related Receptor gamma and Cytochrome CYP19 Aromatase

Estrogen-Related Receptor gamma (ERRγ) is a transcriptional factor acting on mitochondrial biogenesis (see paragraph 2.4.1) and its expression is reported in metabolically active human tissues, including placenta [Takeda *et al.,* 2009]. *ERRγ* showed to be upregulated during cyto- to syncytiotrophoblast cells differentiation in normal culture condition and studied in silencing experiments on trophoblast cells cultured under hypoxic condition, where hypoxia seems to trigger its gene expression [Kumar and Mendelson, 2011]. Moreover, in Luo et al., 2011, ERRy is hypothesized to regulate HIF1α- dependent or independent mechanisms (see paragraph 2.4.3) in response to an hypoxic environment, in a cytotrophoblasts culture model.



Mendelson and colleagues have hypothesized an O2-responsive action for ERRγ which in turn should induce *hCYP19*, the Cytochrome P450 aromatase gene (CYP19, on the side) [Kumar *et al.,* 2011]. Both *ERRγ* e *CYP19* gene expressions seem to be downregulated under hypoxia. CYP19 is an aromatase involved in estradiol production (see paragraph 2.3.3).

Moreover, ERRγ can affect the estrogen pathway by directly binding the Estrogen Receptor (ER) with two different mechanisms:

1) According to Horard hypothesis [Horard and Vanacker, 2003] ERR can act as a transcriptional regulator *per se* or co-binding ER to enhance the cell-specific estrogenic response
2) According to Giguere hypothesis [Giguere, 2002] a functional kindship and/or transcriptional crosstalk between the ERs and ERRs receptors exists, by which ERR could cooperate or compete with ER affecting estrogen pathway, and consequently the estradiol production.

Indeed the 17beta-estradiol, one of the most biologically potent and naturally occurring estrogen (17β-estradiol or E2), may exert its action binding the ER receptors (both their two isoforms ER-α and ER-β) (Katzenellenbogen, 2001). Once estradiol-ligand bounds its placental receptor, ER-α or ER-β, the originated E2-receptor complex acts as a transcriptional factor, which may influence the estrogen response element of specific genes, as CYP19 gene promoter.

2.4.2 Mitochondria Metabolism: Respiration and Chain Complexes

Most notably, the mitochondrion is the site of the electron transport chain and oxidative phosphorylation system that provides the bulk of cellular energy in the form of ATP. Most of the chemical bond energy from the oxidation of fats and carbohydrates is converted to the reducing power of NADH and FADH2 within the mitochondrial matrix. The respiratory apparatus consists of a series of electrogenic proton pumps that convert this reducing potential to an electrochemical proton gradient across the inner membrane (figure 2.9). The electrochemical potential of this gradient is converted via ATP synthase to the high-energy phosphate ATP bonds. Briefly, glucose enters the cells through specific transporters, is phosphorylated by hexokinase, cleaved in two molecules of glyceraldehyde-3-phosphate and finally dephosphorylated by the pyruvate kinase in the presence of ADP to yield pyruvate. These reactions produce two molecules of ATP and are collectively known as glycolysis (which is anaerobic and cytosolic). Pyruvate, the end product of glycolysis, can be then transformed in lactate by the lactate dehydrogenase (in the presence of an excess of NADH+ H+ during poor respiration), or can enter the mitochondria to undergo an oxidative decarboxylation in the presence of coenzymes (NAD+, FAD) to fuel the respiratory chain to consume oxygen and generate ATP and H2O. This second process is known as respiration, and in contrast to glycolysis is aerobic and mitochondrial.

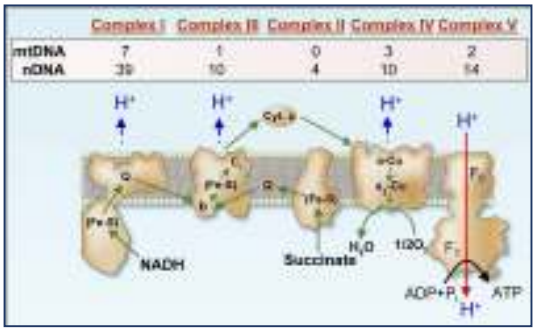


Figure 2.9. Summary of protein subunits of the five respiratory chain complexes, encoded by nuclear and mitochondrial genes. The red arrow shows the flow of protons through complex V from the cytosolic side to the matrix, coupled to the synthesis of ATP. Above each complex the number of protein subunits encoded by nuclear DNA (nDNA) and mtDNA are indicated.

Mt respiratory complexes (complexes I to IV) are responsible for the oxidation of the reducing equivalents, in the form of NADH or FADH2, originating in different metabolic pathways (glycolysis, fatty acid oxidation or the Krebs cycle). Oxidation of NADH and FADH2 is coupled to the pumping of protons into the intermembrane space, and the resulting proton gradient is used by the ATPase (complex V) to generate utilizable energy in the form of ATP. NADH reducing equivalents enter the mitochondrial electron transport chain (mtETC) through complex I, whereas FADH2 reducing equivalents enter the mtETC through complex II or other

dehydrogenases such as electron-transferring-flavoprotein (ETF) dehydrogenase. The electrons are then passed to coenzyme (CoQ), and subsequently to complex III, cytochrome c, and complex IV, which passes them to oxygen as the final acceptor.

The five complexes of the respiratory chain (RC) are embedded within the inner boundary membrane and the cristae membrane. The entire RC complexes system is made by 83 proteic subunits, of which 70 are codified by nuclear DNA (n DNA) and 13 by mitochondrial DNA (mt DNA), as shown in table 1. The inner membrane is impermeable to protons so that a proton electrochemical gradient can be formed upon the proton extrusion from the matrix to the internal membrane space by the respiratory chain complexes I, III and IV (Table 2).

RC Complexes	N° of Complex Subunits	N° of Subunits Codified by n DNA	N° of Subunits Codified by mt DNA
I	41	34	7: *from ND1 to ND6 + ND4L*
II	4	4	none
III	11	10	1: *named Cytocrome B*
IV	13	10	3: *named Cytocrome Oxidase I, II, and III*
V	14	12	2: *named ATPasi 6 and 8*

Table 2: Genetic origin and proteic subunits composition of the mitochondrial respiratory chain complexes. RC: Respiratory Chain; n DNA: nuclear DNA; mt DNA: mitochondrial DNA.

Complex I

In NADH-ubiquinone oxidoreductase or NADH dehydrogenase, two electrons are removed from NADH and transferred to a lipid-soluble carrier ubiquinone (Q). Complex I translocates four protons (H+) across the membrane, thus producing a proton gradient. Complex I is one of the main sites of production of superoxide.

Complex II

In Complex II (succinate dehydrogenase) additional electrons are delivered into the quinone pool (Q) originating from succinate and transferred (via FAD) to Q. Complex II consists of four protein subunits: SDHA, SDHB, SDHC, and SDHD.

Complex III

In Complex III (cytochrome C reductase), the Q-cycle contributes to the proton gradient by an asymmetric absorption/release of protons. When electron transfer is reduced (by a high membrane potential or respiratory inhibitors such as antimycin A), Complex III may leak electrons to oxygen, resulting in superoxide formation.

Complex IV

In Complex IV (cytochrome c oxidase), four electrons are removed from four molecules of cytochrome c and transferred to molecular oxygen, producing two molecules of water. At the same time, four protons are removed from the mitochondrial matrix contributing to the proton gradient. Thus ATP is produced.

Complex V

The efflux of protons from the mitochondrial matrix creates an electrochemical gradient (the already mentioned proton gradient). This gradient is used by the ATP synthase, sometimes described as Complex V, to make ATP via oxidative phosphorylation. Coupling with oxidative phosphorylation is a key step for ATP.

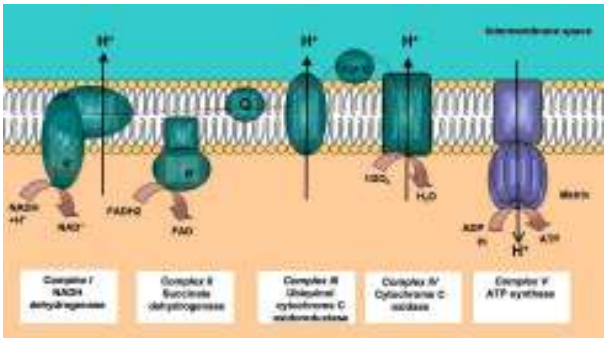


Figure 11: mt respiratory chain [from Bellance, 2009]

The organization of respiratory complexes in the inner membrane has been an object of intense debate. The respiratory components were initially proposed to be closely packed to guarantee accessibility and thus high efficiency in electron transport. However, this original model was progressively abandoned and replaced by the fluid model in which the respiratory complexes are viewed as independent entities embedded in the inner membrane, with CoQ and cytochrome c acting as mobile carriers that freely diffuse in the lipid membrane. However, new evidences from yeast and mammalian allow the reformulation of the solid model proposing that respiratory complexes are organized in larger structures (respiratory supercomplexes, SCs), allowing a more efficient transport of electrons [Genova and Lenaz, 2014].

2.4.3 Mitochondria, IUGR and Hypoxia

Since the establishment of a metabolic link between placenta and mitochondria, a role of mitochondria in growth restriction pathogenesis is well-accepted. They may be responsible of an inadequate oxygen metabolism, whose progressive lack is associated to an increased severity of IUGR phenotype.

Further data have shown an increase of mt DNA content in whole placenta affected by growth restriction compared to controls, with a negative relation between mt DNA levels and the O_2 partial pressure (pO_2) in the umbilical vein [Lattuada et al., 2008]. In a previous study by our group, evaluations of mt DNA content in maternal blood during all the three trimesters of pregnancy, at different gestational ages, resulted in a progressive and significant reduction in pregnant women of I, II, III trimesters and compared to non-pregnant women [Colleoni *et al.,* 2010]. Moreover, in III trimester mt DNA content was found higher in patients with a IUGR fetus compared to women with physiological pregnancies. These data make mt DNA an interesting biomarker to investigate the mechanism behind a physiological or pathological pregnancy As the energy demands of a cell can change dramatically during development and differentiation or due to physiologically changing circumstances, the mitochondrial content is variable and can be adjusted to suit the current situation [Goffart and Wiesner, 2003].

The embryo is highly sensitive to molecular oxygen. The high rate of oxidative metabolism, especially in the second and third trimester, is associated with oxidative and nitrative stress, which in its mild form has been demonstrated in normal placental tissue and is intensified in several pathological conditions [Myatt, 2010; Webster *et al.,* 2008]. Many physiological systems adapt, and are homeostatically regulated, to supply sufficient oxygen to meet energy demands whilst also protecting cells, and particularly mitochondria, from excessive concentrations that could lead to oxidative damage. The invasive form of implantation displayed by the human conceptus presents particular challenges in this respect. In addition, several studies related both acute and chronic hypoxia to mt DNA content changes. In 2008 Gutsaeva and collegues demonstrated that acute hypoxia, affecting the subcortical region of mice brain, activate mitochondrial and nuclear pathways regulating mt biogenesis. Cells respond to this negative *stimulus* through the upregulation of mt duplication and trascription [Gutsaeva *et al.,* 2008]. *In vivo* models show an increase of mt oxygen consumption after chronic exposure to hypoxia [Zung *et al.,* 2007]. Other studies instead demonstrated a downregulation of mt biogenesis, mediated by HIF-1 (hypoxia inducible factor 1), in cancer cell lines. Under hypoxic conditions, HIF-1, which consists of two subunits HIF-1α and HIF-1β, regulates the expression of those

nuclear genes which harbour hypoxia response elements (HRE) on their promoters. Following a three-day exposure to low oxygen, a decrease in HIF-1α mRNA expression was demonstrated in cultured murine ectoplacental cones [Pringle *et al.,* 2007]. HIFs recruit mechanisms to increase oxygen supply (erythropoiesis, angiogenesis, and vasodilation), decrease oxygen demand (increased glycolysis coupled with decreased oxidative metabolism), and regulate cell cycle, apoptosis and autophagy [Semenza, 2000]. *In vitro* studies have demonstrated that hypoxia can affect the proliferation, differentiation, and invasion of cytotrophoblast cells [Caniggia *et al.,* 2002], as well as cause the increase of VEGF transcription and translation in cultured placental fibroblasts [Wheeler *et al*., 1995]. Low oxygen levels also resulted in a shift of the angiopoietin-1: angiopoietin-2 ratio in favour of angiopoietin-2, leading to vessel instability, angiogenesis and vessel remodelling [Zhang *et al.,* 2001].

3 Aim of The Study

The aim of the study is to highlight the complex physiology of placental oxygenation in Intrauterine Growth Restriction (IUGR) fetuses.

The first step was to investigate IUGR and controls placental bioenergetics, by analyzing both mitochondrial content and function in placental tissues and in different placental cell types (cytotrophoblast and mesenchymal stromal cells). Then *in vitro* experiments were performed to assess possible biomolecular mechanisms regulating mithocondrial content in Intrauterine Growth Restriction, by culturing primary placental cells under normal oxygen conditions and hypoxia, a typical feature of IUGR.

4 Materials and Methods

POPULATION

Pregnant patients were enrolled in the Unit of Obstetrics and Gynecology of the L. Sacco Hospital in Milano, Italy. The study was approved by the Institutional Ethics Committee and all pregnant patients gave their informed consent.

Placental samples were collected at cesarean section from physiological at term pregnancies (control group) and IUGR pregnancies. All samples were obtained from non-laboring pregnant women.

IUGR fetuses were identified *in utero* with longitudinal sonographic measurements that showed a reduction below the 5th percentile in fetal growth [Todros *et al.*, 1987]. Depending on gestational age, IUGR were classified according to umbilical arterial Doppler Velocimetry and Fetal Heart Rate (FHR) tracings [Bellotti *et al.,* 2004; Pardi *et al.*, 1993] and divided in 2 different severity groups with normal or abnormal umbilical artery pulsatility index (PI).

Control group had a normal intrauterine growth assessed by longitudinal ultrasound biometry at 20 weeks, 30-32 weeks and term. The newborn weight at term was appropriate for gestational age (AGA) according to Italian standards [Bertino *et al.*, 2010]. Cesarean sections before labor were performed for breech presentation, repeated caesarean section or maternal request. Exclusion criteria were any maternal, placental or fetal disease.

Placental Samples Collection, Tissue Processing and Cytotrophoblasts Isolation

Placentas were collected immediately after cesarean section, weighted and sampled from different sites of the placental disc [Mayhew, 2008] after discarding the cord, the excess of blood and the maternal decidua and collecting the underlying villi. Placental samples - 1cm^3 sized - were washed in PBS (Dulbecco's Phosphate Buffered Solution; Euroclone, Milano, Italy) and temporarily stored in RNAlater solution (Sigma-Aldrich, Saint Louis, MO- USA) at -20°C.

For trophoblast cells processing, 60 g of placental villi were briefly maintained in HBSS 1x (Hank's Balanced Salt Solution) at +4°C. Cytotrophoblast cells were isolated using the trypsin/deoxyribonuclease/dispase/Percoll method [Kliman *et al.*, 1986] and characterized by flow cytometry as cytokeratin 7-positive and vimentin-negative cells. Reagents were supplied by Sigma-Aldrich (HBSS, HEPES, trypsin, Percoll), BD-Becton Dickinson (dispase- Bedford, MA- USA), Roche (DNase- Mannheim, Germany) and Euroclone (gentamicin, Fetal Bovine Serum and Dulbecco's Modified Eagle's Medium-HG, EuroClone, Italy).

4.1 DNA, RNA and protein extraction

Total RNA, DNA, and proteins were isolated from placental tissues, cytotrophoblast cells and p-MSCs using TRIzol reagent (Roche Diagnostics, Indianapolis/IN, USA) following the manufacturer's instructions. To isolate RNA from placental tissues stored in RNAlater, small fragments (90 mg) were added with a TRI reagent solution (Ambion, Austin, TX) and minced with a Potter homogenizer until the mushy solution resulted free of placental pieces; RNA was then extracted by the RiboPure kit (Ambion). RNA was treated with a DNA-free kit (Ambion, Austin, TX) to remove potentially contaminating DNA.

RNA and DNA concentrations were measured by NanoDrop ND1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Protein concentration was determined using the Micro BCA Protein Assay Kit (Thermo Scientific-Pierce Biotechnology, Rockford, IL), according to the manufacturer's protocol.

4.2 Mithocondrial Content and Biogenesis

4.2.1 mt DNA in Placental Tissue and Isolated Cytotrophoblasts

The mtDNA content was measured by Real-Time PCR by normalizing the quantity of a not-polymorphic mitochondrial gene (Cytochrome B) with a single-copy nuclear gene (RNase P). For each gene assay, 30 ng of total DNA were analyzed in triplicate by TaqMan Assay technology, 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). The Sequence Detector software (Applied Biosystems) was used to analyze the data and relative quantification values were calculated according to the $2^{-\Delta Ct}$ method.

4.2.2 NRF-1, ERRγ and CYP19 gene expression

Gene expression of *NRF-1 (NRF1* gene), *ERRγ* (*ESRRG* gene) and *CYP19* (*CYP19A1* gene) was measured by Real-Time PCR in both **placental tissue** and **cytotrophoblast cells**. *NRF1* gene expression was analyzed also in **p-MSCs**.

For each sample, 1.6 µg of total RNA were reverse-transcribed by the High Capacity cDNA Reverse Transcription Kit and the obtained cDNA served as template for quantitative Real-Time PCR with TaqMan assays. Gene expression levels were calculated using the geNorm method [Vandesompele, 2002] relative to *HPRT* (hypoxanthine-guanine phosphoribosyltransferase) and *YWHAZ* (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein-zeta polypeptide) genes for placental tissues and cytotrophoblast cells, and to *PPIA* (*peptidylprolyl*

isomerase A) and *RPL13A* (*ribosomal protein L13a*) genes for p-MSCs. All samples were reverse-transcribed in duplicate and cDNA was run in triplicate to allow assessment of sample homogeneity and technical variability. All reagents were supplied by Life Technologies (Foster City/CA- USA).

4.3 Mitochondria Metabolism: Respiratory Chain Efficiency in IUGR versus Controls

4.3.1 Respiratory Chain complexes gene and protein expression

We measured by Real-Time PCR mRNA levels of four subunits belonging to four respiratory chain complexes (RCC) in the **cytotrophoblast cells** isolated from 8 control, 6 PE and 8 IUGR placentas. Analyzed genes were: NADH-dehydrogenase-1-alpha subcomplex 9 (NDUFA9, complex I- CI), succinate dehydrogenase complex-subunit A (SDHA, complex II- CII), ubiquinol-cytochrome c reductase core protein I (UQCRC1, complex III- CIII), cytochrome c oxidase subunit IV isoform 1 (COX4I1, complex IV- CIV). *UQCRC1* and *COX4I1* genes were also analyzed in p-MSCs.

For each sample, 1.6 µg of total RNA were reverse-transcribed by the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and obtained cDNA served as template for quantitative Real Time PCR with TaqMan assays (Applied Biosystems). Gene expression levels were calculated using the geNorm method relative to HPRT and YWHAZ genes for cytotrophoblast cells, and to PPIA and RPL13A genes for p-MSCs. All samples were reverse-transcribed in duplicate and cDNA was run in triplicate to allow assessment of sample homogeneity and technical variability.

Western blotting was performed on proteins extracted from the same cytotrophoblast cells used for gene expression analysis. Proteins from each sample (50 µg) were precipitated overnight (ON) at -20°C in 2.5 V of acetone and resolved under reducing conditions by SDS polyacrylamide gel electrophoresis. Separated proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (iBlot® Dry Blotting System; Invitrogen). All non-specific bindings were blocked by membrane incubation in a blocking buffer (5% skimmed powdered milk in TBS-T [TBS, 0.3% Tween 20]) for 2.5 hours at room temperature. Membranes were incubated ON in blocking buffer with mouse monoclonal antibodies against the four RCC subunits analyzed by Real Time PCR (1:5000 dilution; Molecular Probes, Invitrogen). After incubation with an anti-mouse HRP-conjugated antibody, protein bands were detected by enhanced chemiluminescence method (ImmunStar Western C; Bio-Rad, Hercules, CA). All membranes

were subsequently stripped and reprobed with anti-β-actin antibody (1:15000 dilution; Sigma-Aldrich). Bands intensity was measured by ImageJ software (freely available at http://rsbweb.nih.gov/ij/). For relative semi-quantitative analysis, the protein expression values were normalized on β-actin. Data from each blot were referred to a sample chosen as reference to allow comparison among different experiments.

4.3.2 Oxygen consumption - High-Resolution Respirometry

The Respiratory Chain Complexes (RCC- complexes CI-CII-CIII-CIV) efficiency was determined by high resolution respirometry (HRR) on **cytotrophoblast cells** isolated from ten placentas of the IUGR group and seven of control group. This technique allows to measure total mitochondrial (mt) O_2 consumption and to assess the role of each mt complexes in the process. O_2 suspension was measured by high resolution respirometry with an Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria) in MiR06 medium at 37°C (0.5 mM EGTA, 3 mM $MgCl_2$*6 H_2O, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose, 1 g/l BSA, 280 µg/ml catalase) according to HRR MipNEt protocols (Oroboros Instruments). MiR06 was equilibrated and then replaced by the cell suspension, which was continuously stirring at 460-600 rpm.

The experimental protocol started with Routine Respiration, defined as the O_2 consumption of cell suspension without any substrates addition. Cells were then permeabilized with 40 µg/ml digitonin allowing loss of plasma membrane barrier, but maintaining intracellular membrane structure (mitochondria and endoplasmatic reticle). Complex I was measured by the injection of glutamate (10 mM) and malate (2 mM) substrates in presence of 2.5 mM ADP. Cytochrome C test was performed to assess the intactness of mt outer membrane, since in case of damage it would be released with a consequent inhibition of mt respiration. Addition of 10 mM Succinate, in presence of conventional substrates for CI, supported the simultaneous convergent electron flow through CI+II into the Q-junction. The convergent electron flow mimick the action of the tricarboxylic acid cycle in the intact cell, which generates both NADH and Succinate in the matrix as substrates for CI+II. Complex II was measured by injecting 0.5 µM Rotenone and inhibiting Complex I after convergent stimulation of CI+II. Finally, inhibition of complex III with 2.5 µM Antimycin-A and of complex II with 5 mM Malonate was followed by Complex IV stimulation with 0.5 mM TMPD (N,N,N',N'-Tetramethyl-p-Phenylenediamine) and 2 mM Ascorbate.

DatLab software (Oroboros Instruments) was used for data recording and analysis (1 or 2 s time intervals), calculating the time derivative of O_2 concentration, adjusted for instrumental background. The O_2 consumption levels per-mitochondrion were obtained by normalizing data to the cytotrophoblast mtDNA levels.

4.4 Placental Primary Cells Culture

4.4.1 Cytotrophoblast Cells Culture under Hypoxia

Primary trophoblast cells were isolated from human placentas (n= 3) of term singleton uncomplicated pregnancies of non-smoking women with appropriately grown fetuses (AGA) and no maternal/fetal pathologies.

The collected placental samples were processed with a mechanical disgregation (as described in paragraph 4.1) followed by an enzymatic tissue digestion (dispase/DNAse enzymes). Villous cytotrophoblasts were selected through a stratification by Percoll gradient, as previously described, and a cytofluorimetry characterization (cytokeratin 7^+; vimentin$^-$).

Characterization of Cytotrophoblast Cells by Flow-Citometry

Isolated cells from physiological placentas were characterized by flow-cytometry. The samples were labelled with a Mouse anti-human cytokeratin 7 (CK7) e and a Mouse anti-human Vimentin (VIM) (Dako), two mutually exclusive intra-cytoplasmatic primary antibodies with an higher specificity cytotrofoblast [Maldonado-Estrada et al., 2004]. For secondary labelling, an Anti-Mouse IgG FITC (eBioscience) was used. An endogenous and an isotype controls were analysed together with the studied samples. The isotype control is useful to assess the level of background staining in all cell-antibody binding assays (table 4.4.1).

Antibody	Clone	Specificity
Mouse anti-human CK7 (Dako)	OV/TL 12/30 (IgG1)	Cytokeratin 45 kDa
Controllo Isotipico (Dako)	IgG1	Non-specific isotype control
Mouse anti-human Vimentin (Dako)	Vim3B4 (IgG2α)	Mesenchymal Cells 57 kDa
Anti-Mouse IgG FITC (eBioscience)	F(ab')2	Fab fragment of Mouse IgG

Tabella 4.4.1: Flow-Citometry Antibodies to select cytotrophoblast cells

Cytotrophoblast Cells Culture under Normal or Hypoxic Conditions

Isolated cytotrophoblast cells were then plated (300 cells/cm^2) in 2,4 ml of HG-DMEM medium with 200 mM L-glutammine, 10 mg/ml Gentamicin, 10% FBS in standard growth conditions

(20% O_2; 5% CO_2; 37C°) for 4 hours (h) to allow their adhesion and growth. After this time, cells were shifted in different O_2 conditions:

- ✓ **20% O_2**- standard growth (20% O_2; 5% CO_2; 37C°). At this condition, cells were cultured with normal medium with/without the addition of 0,2 mM Cobalt Chloride ($CoCl_2$), an activator of HIF1α, for chemical induction of hypoxia;
- ✓ **8% O_2**- oxygen conditions that mimics 2^{nd}-3^{rd} trimester placentas oxygenation (Galaxy 48R Incubator);
- ✓ **0.1% O_2** hypoxic condition (Ruskinn Cabin).

Cells were grown under hypoxic condition in a RUSKINN CABIN INVIVO2 200. Trophoblasts were cultured for 72 h and frozen at T24, T48, T72. Medium was refreshed every 24 hours.
the culture media was re-freshed every 24 h.
mtDNA content will be assessed as described in paragraph 4.2.1.

hCG levels in Culture Media: trace of Syncytialization

Human Chorionic Gonadotropin (hCG) is a glycoprotein made by two subunits α and β. In the placental tissue is released by syncytiotrophoblast cells. hCG placental production reflects the histological and morphological changes during the maturation of the human placenta. Thus its concentration is a marker of the occurring differentiation affecting the cytotrophoblast cells changing into syncytiotrophoblast. [hCG] was measured in the culture media (ImmunoAssay Eclia) with specific monoclonal antibodies labelling both hCG and free circulating β form.

4.4.2 p-MSCs Culture: Mesenchymal Stromal Cells from Physiological and IUGR Placentas

p-MSCs Culture and Expansion

Placental tissue was collected immediately after cesarean section; 1.5-cm^3 full-thickness pieces were sampled in different sites of the placental disc and washed in HBSS (Hank's balanced salt solution; Sigma-Aldrich, St. Louis, MO, USA). After mechanical separation of fetal membranes from the placental basal disc, the tissues were enzymatically digested with Collagenase IA (Gibco, Life Technologies) and Trypsin 2,5% (Gibco) and incubated in a fully humidified atmosphere of 5% CO_2, 95% air at 37°C for 45 minutes. Tissues were then filtered and centrifuged at 2000 rpm for 10 minutes and cells grown in an expansion medium, as previously described [Jaramillo-Ferrada 2012] with the following minor modifications. Human p-MSCs,

isolated from physiological and IUGR placentas, were plated in 6-well tissue culture plates (International PBI, Milano, Italy) and coated with 0.2% gelatin (Sigma-Aldrich) at a density of 104 cells/well. Cells were grown in expansion medium composed of DMEM/F-12 (1:1) (Life Technologies, Grand Island, NY, USA), 10% FBS and 20 ng/ml EGF (Miltenyi Biotec, Bergisch Gladbach, Germany). Expansion culture media were prepared fresh weekly. Cells were incubated in a fully humidified atmosphere of 5% CO_2, 95% air at 37°C for several weeks. Every week, living cells were counted with a Burker chamber using Trypan Blue (Sigma-Aldrich).

p-MSCs FACS analysis

At 24 hours, 7 days and 30 days, 105 cells were incubated with the following antibodies: anti-CD133-PE, anti-CD34-APC and anti-CD117-PE (hematopoietic stem cell markers), anti-CD31-FITC (endothelial cell marker), anti-CD45-APC-CY7 (hematopoietic cell marker), anti-CD44-FITC, anti-CD105-PE, anti-CD29-FITC, anti-CD73-APC and anti-CD90-PE-Cy5 (mesenchymal cell markers) (Miltenyi Biotec) and anti-7-amino-actinomycin D (anti-7AAD, used to analyze only alive cells; BD Biosciences-Pharmingen, San Diego, CA, USA). Isotype-matched mouse immunoglobulins were used as negative controls. After each incubation, performed at 4°C for 20 minutes, cells were washed in PBS, 1% heat-inactivated FCS and 0.1% sodium azide. Cytometric analyses were performed with a Cytomics FC 500 flow cytometer and 2.1 cxp software (BC, Beckman Coulter, Miami, FL, USA). Each analysis included at least 10,000-20,000 events for each gate. A light-scatter gate was set up to eliminate cell debris from the analysis. The percentage of positive cells was assessed after the correction for the percentage reactive to an equivalent isotype control.

p-MSCs In Vitro Differentiation

Endothelial differentiation

Post-expansion Human p-MSCs were incubated in an endothelial growth medium containing Medium 199 (M199; Gibco-BRL, Gaithersburg, MD, USA) supplemented with 20 ng/ml EGF, 10 ng/ml VEGF and 5 units/ml heparin (Sigma-Aldrich). Cells were seeded on 48-well plates coated with Matrigel/199 (1:1) and incubated in a humidified atmosphere (5% CO_2, 95% air at 37°C). Differentiated endothelial cells were detected by immunostaining with anti-Factor VIII antibody (1:100 dilution; DAKO, Glostrup, Denmark). Nuclei were counterstained with DAPI. Images were captured using a fluorescence microscope (Leica DMIRE2; Leica Microsystems). Endothelial differentiation was quantitatively evaluated by counting FactorVIII-positive cells

per microscope field. The endothelial differentiation capacity of p-MSCs in 3D culture assay was also assessed and 10^5 p-MSCs were seeded in 24-well plates containing matrigel matrix (Becton Dickinson BD, California, USA) and repeatedly observed after 24, 48 and 72 hours of culture.

Adipogenic differentiation

Adipogenic potential of p-MSCs was verified using a specific differentiation kit (hMSC Adipogenic BulletKit; Lonza, Basel, Switzerland). Histological (Oil Red O; Sigma-Aldrich) and immunofluorescence (FAB4, Perilipin A; Abcam) stainings were performed to confirm differentiation; nuclei were stained with hematoxylin. Histological pictures were acquired with an optical microscope, while, for the immunostainings, an inverted fluorescence microscope was used (Leica DMIRE2; Leica Microsystems). Oil Red O staining was then eluted with isopropanol from cell vacuoles; optical density of the samples was measured at 490nm (GloMax Discover - Promega, Madison, WI, USA) using pure isopropanol as control.

4.5 Biochemical analyses

Umbilical venous and arterial blood was sampled from a segment of the doubly clamped umbilical cord immediately after fetal extraction. All samples were collected in heparinized syringes and kept on ice until the end of analysis. Standard biochemical parameters, such as oxygen partial pressure and saturation, CO_2 partial pressure, lactate concentration, hemoglobin and pH values were immediately measured by a GEM Premier 3000 analyzer (Instrumentation Laboratory, Brussels- Belgium).

4.6 Statistical analyses

Clinical characteristics of the population, FACS data, quantitative misures of differentiation abilities, mtDNA, mRNA and protein expression levels, oxygen consumption and biochemical data were compared among groups using independent-samples t-test. Levene's test for equality of variances was performed to assume that samples were obtained from populations of equal variances. P-MSCs proliferation behavior was analyzed with 2-way ANOVA. Differences between cases and controls were considered statistically significant when $p < 0.05$ or $p < 0.017$, if the Bonferroni adjustment was applied to t-test.

The correlation between values was analyzed using the Spearman correlation and considered significant when $p < 0.05$. All tests were performed using the statistical package SPSS (IBM SPSS Statistics, Armonk, NY, NY).

5 Results

1 Mt Content and Biogenesis in IUGR versus control placentas and isolated trophoblast cells

Clinical data of the study population

Maternal and fetal baseline demographic data of cases and controls are compared in **table 1.1A**. Maternal age and BMI are not significantly different in the two groups. As expected, gestational age and fetal and placental weights are significantly lower in IUGR group (n=8) *versus* controls (n=8). **table 1.1B** shows clinical and diagnostic characteristics of each single case.

	Controls (n = 8)	IUGR (n = 8)
Maternal Age (yr)	34.1 ± 2.6	34.9 ± 2.0
Pre-Pregnancy BMI (kg/m²)	21.6 ± 1.1	23.1 ± 1.3
Gestational Age (wk)	39.1 ± 0.1	32.7 ± 1.3**
Placental Weight (g)	517.2 ± 28.2**	239.6 ± 34.5**
Fetal Weight (g)	3398.7 ± 88.7**	1386.7 ± 186.1**

Table 1.1A: Maternal and fetal demographic data in IUGR *vs* controls. Data presented as average±SE *p<0.017 vs controls: **p<0.001 vs controls.

	Fetal Sex	Gestational Age (weeks)	Placental Weight (g)	Fetal Weight (g)	Neonatal Weigth (percentile)	UA Pulsatility Index (PI_{umb})
IUGR	M	26	100	707	<10°	Abnormal
IUGR	F	27	135	640	<10°	Abnormal
IUGR	F	32	161	1030	<5°	Abnormal
IUGR	F	33	235	1252	<10°	Abnormal
IUGR	F	34	340	1550	<10°	Abnormal
IUGR	F	35	276	1745	<5°	Normal
IUGR	M	35	350	2005	<10°	Normal
IUGR	F	37	320	1965	<5°	Normal
Control	F	39	161	3220	50°-75°	Normal
Control	F	39	135	3105	25°-50°	Normal
Control	F	39	235	3630	75°-90°	Normal
Control	F	40	340	3845	75°-90°	Normal
Control	M	39	100	3250	25°-50°	Normal
Control	M	39	350	3550	50°-75°	Normal
Control	F	39	276	3335	25°-50°	Normal
Control	F	39	320	3255	25°-50°	Normal

Table 1.1B: Prenatal and neonatal baseline data in cases and controls. Umbilical Artery (UA) pulsatility index (PI) by Doppler Velocimetry. IUGR, intrauterine growth restricted fetuses; A, abnormal; N, normal. Data presented as average ± standard deviation (SE).

Mt DNA in Placental Tissue and Cytotrophoblast Cells

mtDNA levels are significantly higher in IUGR placental tissues (291.5 ± 132.8) compared to controls (179.7 ± 62.0) (p=0.05, **figure 1.1A**). mtDNA levels are instead significantly lower in IUGR cytotrophoblasts (318.0 ± 118.1) compared to controls (495.7 ± 105.7) (p=0.007, **figure 1.1B).**

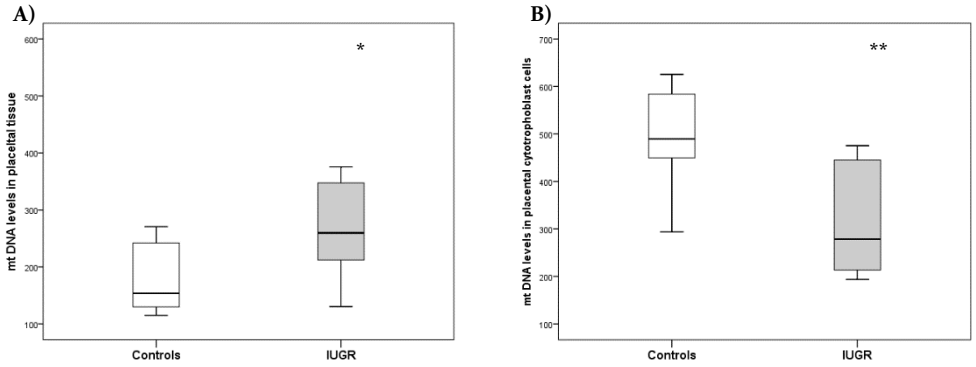


Figure 1.1: Mt DNA content in A) placental tissue and B) cytotrophoblast cells: mt DNA levels in controls (n=8) and IUGR (n=8). Data shown as BoxPlots graphs. Mt DNA levels calculated according to 2^-ΔCt method. Statistical analysis by Student's T-test: *p=0.05; **p<0.01 *vs* controls.

NRF1 Expression in Placental Tissue and Cytotrophoblast Cells

NRF1 mRNA levels in placental tissue are on average 24% higher in IUGR (0.57 ± 0.12, n=6) compared to controls (0.47 ± 0.12, n=5), however the difference is not statistically significant (**figure 1.2A**). On the contrary, NRF1 gene expression is significantly lower in IUGR cytotrophoblast cells (0.46 ± 0.08, n=6), with a 30% expression decrease compared to controls (0.66 ± 0.20, n=5) (p=0.05, **figure 1.2B**).

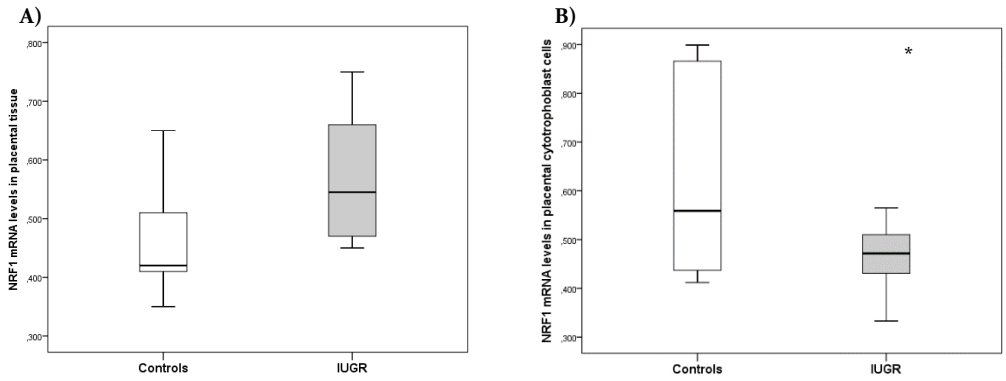


Figure 1.2: Nuclear Respiratory Factor-1 (NRF-1) mRNA levels in placental tissue A) of controls (n=5) and IUGR (n=6) and in cytotrophoblast cells B) of controls (n=6) and IUGR (n=5) pregnancies. Relative mRNA levels calculated with geNorm method, by E^-ΔCt/NF, where E is the efficiency of each assay and NF is the normalization factor. Statistical analysis by Student's T-test: *p=0.05 versus controls.

2 Mt Respiratory Chain (RC) Complexes Expression and O_2 Consumption

RC Complexes Gene and Protein Expression in Cytotrophoblast Cells

mRNA expression of CII, CIII and CIV in cytotrophoblast cells of IUGR (n=8) is lower than in controls (n=8), though this difference do not reach statistical significance when corrected by the Bonferroni adjustment (p=0.04) (**figure 2.1A**).

The protein expression of the same subunits is not significantly different between cytotrophoblast cells of IUGR group compared to controls (**figure 2.1B**).

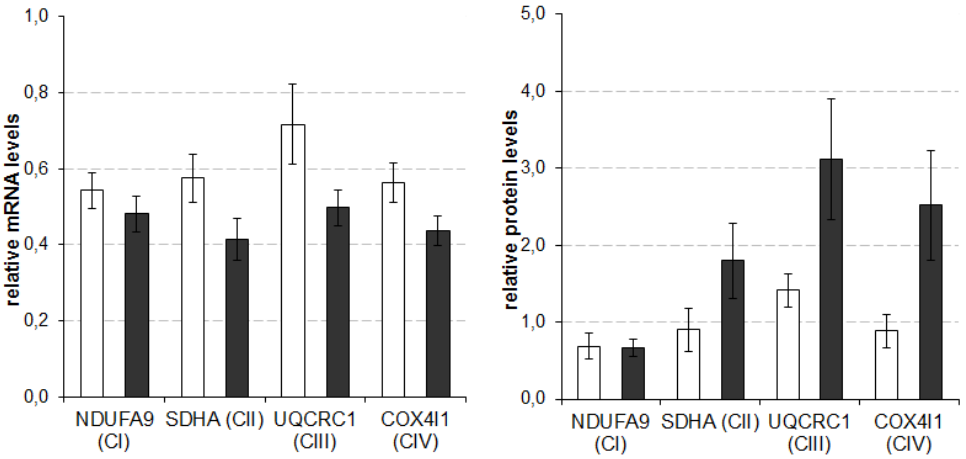


Figure 2.1. mRNA A) and protein B) expression of the subunits belonging to four respiratory chain complexes in the control (n=8, white) and IUGR (n=8, dark gray) pregnancies. Graphs represent average*standard deviation (SE). Relative mRNA levels calculated as for NRF-1 gene expression. Western blotting protein levels were assessed by band intensities measured by ImageJ software, normalized on β-actin band intensities.

Cytotrophoblast Cells O_2 Consumption

Figures 2.2A and 2.2B show the O2 consumption levels per-mitochondrion (2.2A) and per-number of cells (2.2B) resulting from the activity of the respiratory chain complexes. Cytotrophoblast cells of the IUGR group show significantly higher coupled-O2 consumption compared to controls, both as total assessment (CI+CII) and at the level of the single respiratory complex CIV, normalized to mitochondrial content (p< 0.017; **figure 2.2A**).

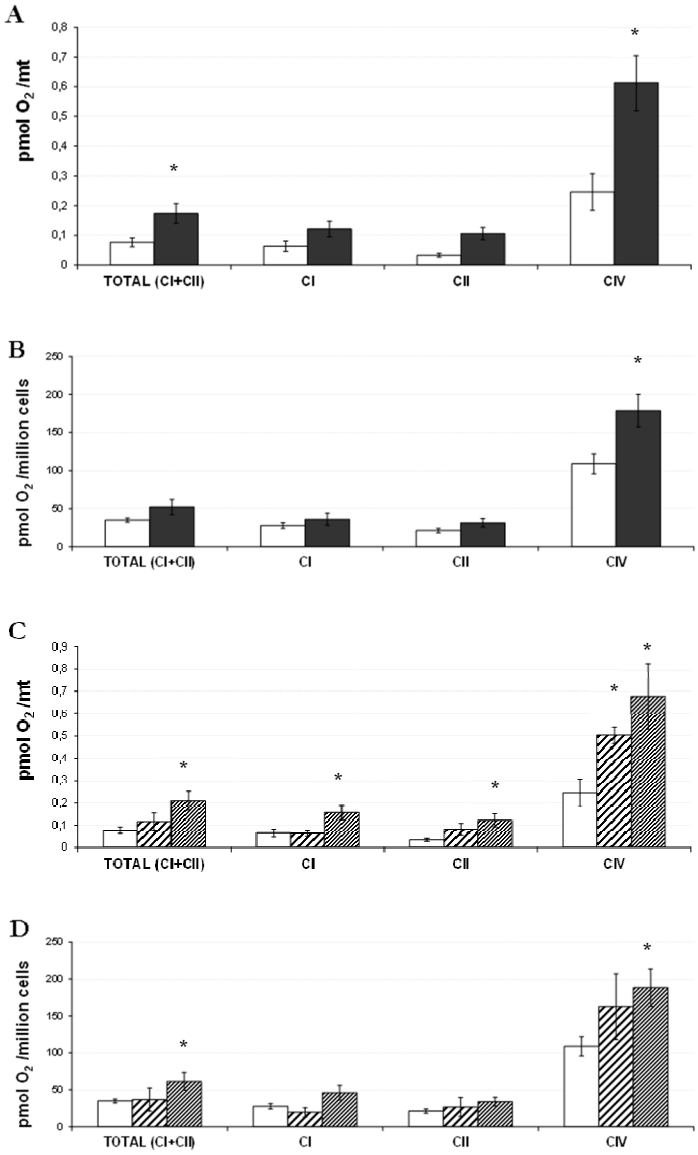


Figure 2.2: Oxygen consumption levels (pmol O_2) per mitochondrion (A and C) and per million of cells (B and D) in cytotrophoblast cells isolated from placentas of controls (white bars) and A-B) all IUGR cases (dark gray bars); C-D) IUGR with normal umbilical PI (hatched bars) and IUGR with abnormal PI (crosshatched bars). Graphs represent average values * standard deviation (SE). *p< 0.017 adjusted to Bonferroni Test.

Interestingly, data not normalized to mtDNA show higher CIV O_2 consumption in cells from IUGR placentas, notwithstanding their lower content of mtDNA (**figure 2.2B**).

In IUGR cases with abnormal umbilical PI (PIumb), O_2 consumption per-mitochondrion is higher than controls for all reported complexes (**figure 2.2C**). This resulted in higher CIV and total O_2 consumption also overall in the cell (**figure 2.2D**). IUGR cases with normal PIumb are instead similar to controls except for an increased per-mitochondrion respiration in complex IV, that however do not reflect on the cell overall total respiration. No gender difference for O_2 consumption levels was found (data not shown).

Data Correlations

In our population mt DNA content in placental tissue inversely correlates both with fetal (R^2=0.2; p=0.03) and placental (R^2=0.3; p=0.008) weight (**figures 2.3A and B**). The mt DNA levels of both placental tissue and isolated cytotrophoblast cells do not correlate with gestational age (data not shown). Trophoblast cells mt DNA inversely correlates with normalized total (CI+CII) O_2 consumption (R^2=0.3; p=0.02); this is sustained by the single respiratory complexes (CI: R^2=0.3; p=0.02. CIV: R^2=0.6; p<0.001) (**figures 2.4A, B and C**).

Both fetal and placental weights inversely correlate with cytotrophoblast mitochondrial O_2 consumption (data not shown).

Finally, total O_2 consumption values, regardless of their normalization on mt DNA or cell number, inversely correlate with gestational age (data not shown).

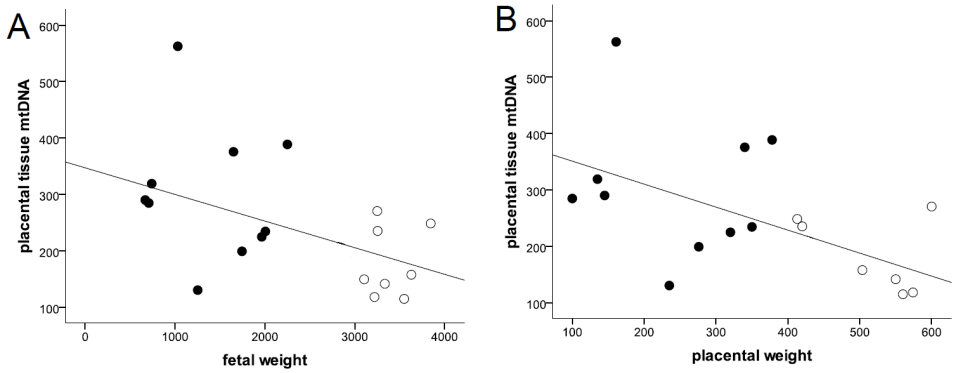


Figure. 2.3:. Significant correlations between placental mt DNA content and fetal A) and placental B) weight in the analyzed population of controls (n=8, ○) and IUGR (n=8, ●) pregnancies. Statistical Analyses by Pearson Correlation: A) p=0.03/R^2=0.2; B) p=0.008/R^2=0.3.

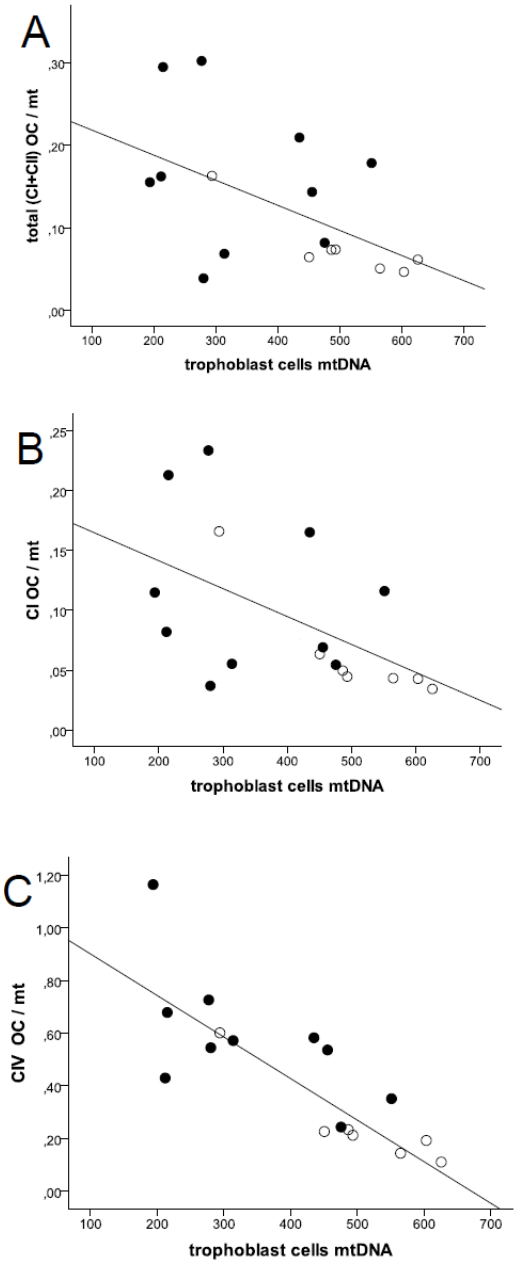


Figure 2.4: Significant correlations with oxygen consumption levels of cytotrophoblast cells in the analyzed population of controls (n=7, ○) and IUGR (8, ●) pregnancies. Correlations between cytotrophoblast cell mt DNA content and A) total, B) CI, and C) CIV oxygen consumption levels per mitochondrion. Statistical Analyses by Pearson Correlation: A) $p=0.02/R^2=0.3$; B) $p=0.02/R^2=0.3$;. C) $p<0.001/R^2=0.6$.

3 ERRγ and CYP19

Clinical Data of the Study Population

Maternal and fetal clinical data are reported in **table 3.1.** Maternal age and BMI do not differ between IUGRs and control group, while gestational age, placental and fetal weight are significantly lower in IUGR (p<0.001), as expected.

	Controls (n = 17)	IUGR with normal PI (n = 8)	IUGR with abnormal PI (n = 9)
Maternal Age (yr)	34.59 ± 5.54	35.87 ± 4.40	34.00 ± 5.92
Pre-Pregnancy BMI (kg/m²)	21.31 ± 2.99	21.49 ± 2.15	24.26 ± 4.17
Gestational Age (wk)	39.18 ± 0.37	34.95 ± 3.64***	32.62 ± 3.59***
Placental Weight (g)	467.66 ± 84.11	315.87 ± 128.86***	243.43 ± 128.21***
Fetal Weight (g)	3367.94 ± 323.79	1767.75 ± 648.35***	1500.68± 784.71***

Table 3.1: Maternal and fetal data of physiological (controls) and IUGR pregnancies. Data showed as average ± standard deviation. *p<0.05, ***p<0.001.

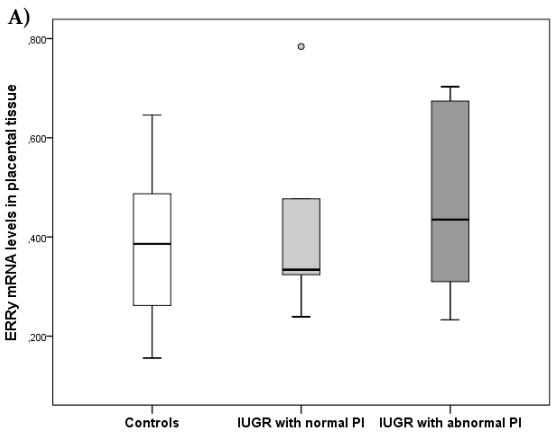
ERRγ and CYP19 Gene Expression in Placental Tissue

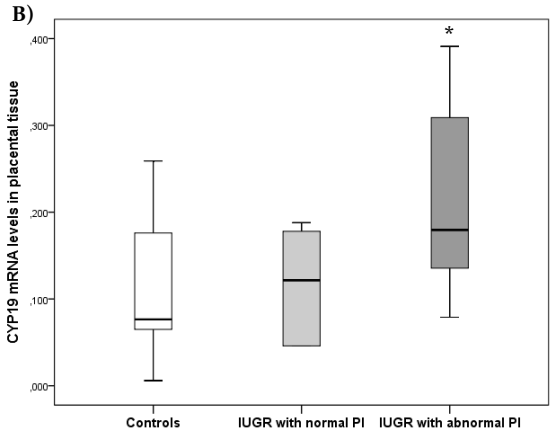
ERRγ levels tend to increase in IUGR (normal PI: 0.43 ± 0.21, n=5; abnormal PI: 0.47 ± 0.19, n=9) compared to control term placentas (0.34 ± 0.14, n=14), although not significantly (**Figure 3.1A**). *CYP19* gene expression shows no differences between IUGR (0.17 ± 0.10, n=14) and controls (0.11 ± 0.08, n=14), although it positively correlates with *ERRγ* expression (see Data Correlations paragraph). However, when analyzing IUGR sub-populations according to disease severity, *CYP19* expression results significantly different with a 33% increase in most severe IUGR cases (abnormal PI: 0.22 ± 0.11, n=8) compared to controls (0.11 ± 0.08, n=14; p=0.036, **figure 3.1B**).

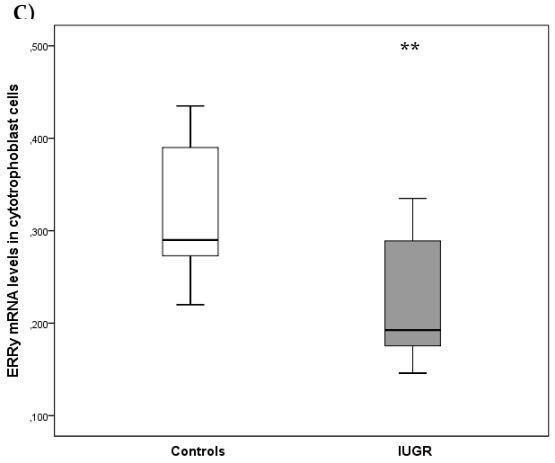
ERRγ and CYP19 Gene Expression in Cytotrophoblast Cells

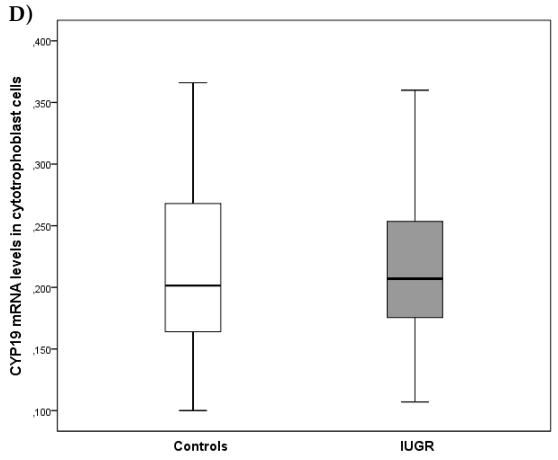
ERRγ expression levels results significantly lower in IUGR cytotrophoblast cells (0.22 ± 0.71, n=8) compared to controls (0.32 ± 0.07, n=10) (p=0.016, **figure 3.1C**). When analyzed in IUGR sub-populations according to disease severity, *ERRγ* is significantly lower in the most severe group, with abnormal PI, with a 28% decrease compared to controls (p=0.007, data not shown). *CYP19* expression in cytotrophoblast cells shows no differences between the two groups (**figure 3.1D**), but presents a significant correlation with *ERRγ* measured in the same placentas, as shown in **figure 3.2B** (see Data Correlations paragraph).

Figure 3.1: *ERRγ* and *CYP19* mRNA levels in placental tissue. A) *ERRγ* mRNA expression in controls (n=14) and IUGR with normal (n=5) or abnormal (n=9) PI; B): *CYP19* expression in controls (n=14) and IUGR with normal (n=6) or abnormal (n=8) PI. *ERRγ* and *CYP19* mRNA levels in placental cytotrophoblast cells. C) *ERRγ* expression in controls (n=10) and IUGR (n=8) cytotrophoblasts. D) *CYP19* expression in controls (n=10) and IUGR (n=8) cytotrophoblasts. Data shown as BoxPlots graphs. Statistical analysis by Student's T-test: *p<0.05; **p<0.01 *versus* controls. Relative mRNA levels using the geNorm method, by $E^{-deltaCT}$/NF, where E is the efficiency of each assay (calculated by a calibration curve) and NF is the normalization factor (calculated by housekeeping gene values)


A)
ERRγ mRNA levels in placental tissue
,800
,600
,400
,200
Controls
IUGR with normal PI
IUGR with abnormal PI


B)
CYP19 mRNA levels in placental tissue
*
,400
,300
,200
,100
,000
Controls
IUGR with normal PI
IUGR with abnormal PI


C)
ERRγ mRNA levels in cytotrophoblast cells
**
,500
,400
,300
,200
,100
Controls
IUGR


D)
CYP19 mRNA levels in cytotrophoblast cells
,400
,350
,300
,250
,200
,150
,100
Controls
IUGR

Data Correlations

In our study population, *ERRγ* positively correlates with *CYP19* gene expression both in whole placental tissue and isolated trophoblast cells (placental tissue: p=0.012/R^2=0.247, **figure 3.2A**; trophoblast cells: p=0.037/R^2=0.244, **figure 3.2B**).

Expression levels of both analyzed genes do not correlate with gestational age, except for *CYP19* mRNA in whole placenta (p=0.023/R^2=0.184, data not shown). Moreover, *ERRγ* and *CYP19* expressions are significantly related to maternal BMI, with a positive trend in placental tissue and a negative one in trophoblast cells isolated from the same placentas (placental tissue: p=0.041/R^2=0.157 for both *ERRγ* and *CYP19,* **figures 3.3A-B**; trophoblast cells: p=0.021/R^2=0.289 for *ERRγ* and p=0.020/R^2=0.269 for *CYP19,* **figures 3.3C-D**).

Finally, in the analyzed population the Umbilical Vein (UV) lactate content is positively correlated with *CYP19* levels in placental tissue, and negatively related with *ERRγ* in cytotrophoblast cells (p=0.018/R^2=0.318 and p=0.021/R^2=0.463, respectively) (data not shown).

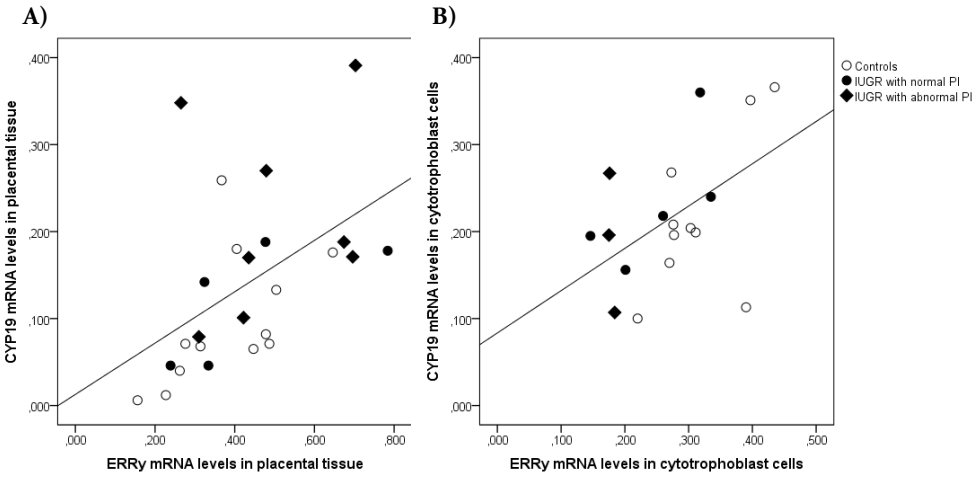


Figure 3.2: Significant correlation between placental *ERRγ* and *CY19* mRNA expression in A) whole placental tissue (Controls: n=14, ○; IUGR with normal PI: n=5, ● and with abnormal PI: n=8, ◆) [p=0.012/R^2=0.247]; B) cytotrophoblast cells (Controls: n=10, ○; IUGR with normal PI: n=5, ● or with abnormal PI: n=3, ◆). Statistical Analyses by Pearson Correlation: p=0.037/R^2=0.244

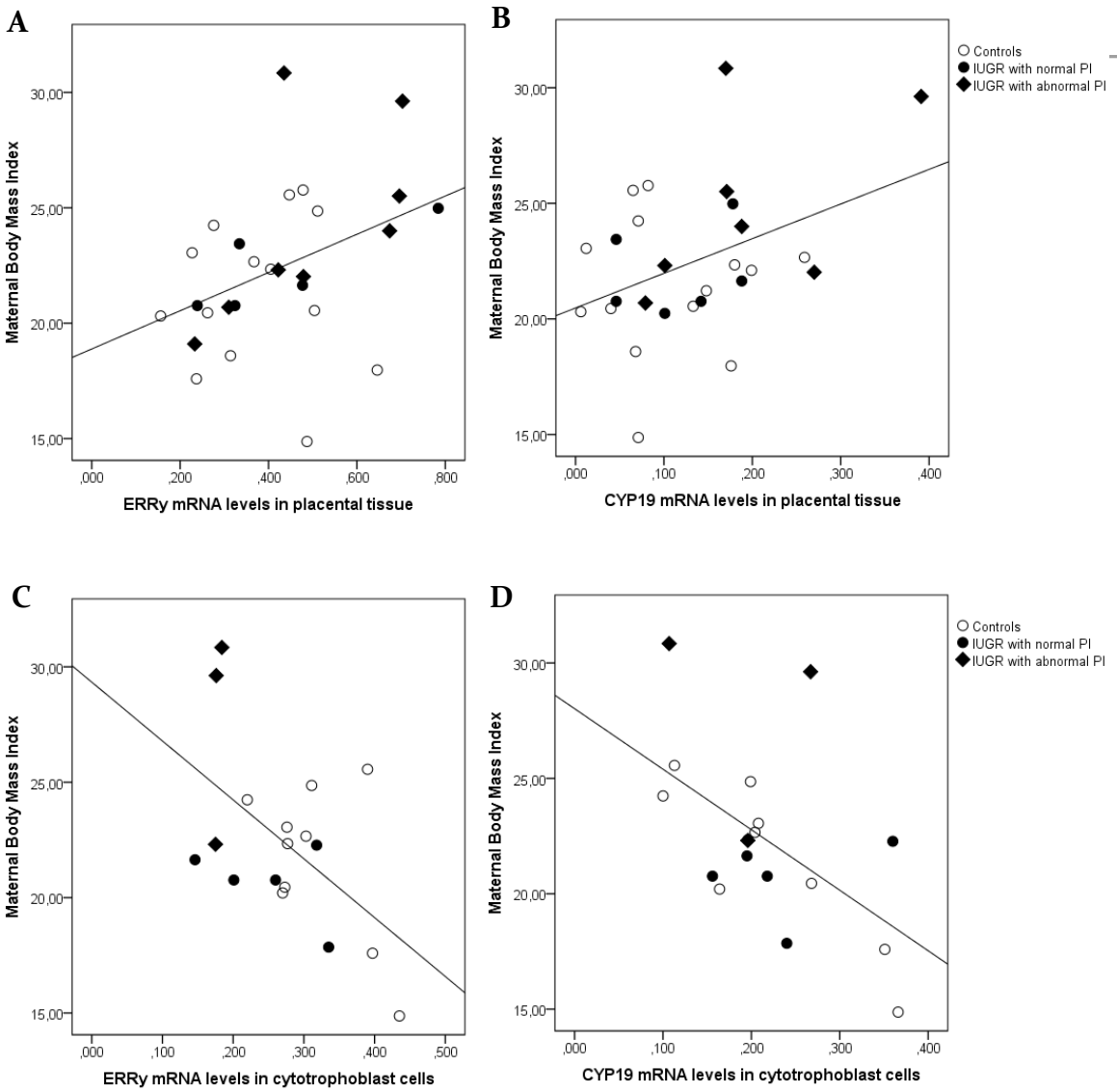


Figure 3.3: Correlations between *ERRγ* or *CYP19* mRNA expression and Maternal Body Mass Index (BMI). Top: significant correlation in whole placental tissue between maternal Body Mass Index (BMI) and A) *ERRγ* in controls (n=14, ○), IUGR with normal (n=5, ●) or abnormal (n=9, ◆) PI, B) *CYP19* in controls (n=14, ○), IUGR with normal (n=6, ●) or abnormal (n=8, ◆) PI. Bottom: significant correlation in cytotrophoblast cells between maternal BMI and C) *ERRγ* in controls (n=10, ○), IUGR with normal (n=5, ●) or abnormal (n=3, ◆) PI, D) *CYP19* in controls (n=10, ○), IUGR with normal (n=5, ●) or abnormal (n=3, ◆) PI. Statistical Analyses by Pearson Correlation: A-B) $p=0.041/R^2=0.157$, C) $p=0.021/R^2=0.289$, D) $p=0.020/R^2=0.269$.

4 Placental Primary Cell Culture: Cytotrophoblast and Mesenchymal Stromal Cells

4.1 Cytotrophoblast Cells Hypoxic Culture Model

Clinical Data of the Study Population

Data are presented in **table 4.1A**. No significant differences were found between groups for maternal age and pre-pregnancy BMI. As expected, gestational age, placental and fetal weights are significantly lower in IUGR compared to physiological pregnancies.

	Maternal Age (yr)	Pre-Pregnancy BMI (kg/m²)	Gestational Age (weeks)	Placental Weight (g)	Fetal Weight (g)
Term Controls (n = 3)	33.0 ± 3.0	21.0 ± 4.0	39.1 ± 0.0	427.0 ± 40.0	3332.0 ± 116.0

Table 4.1A: Maternal and fetal data of physiological pregnancies and IUGR cases. Data are presented as average ± standard deviation. *p<0.05, **p<0.01.

Evaluation of hCG Concentration

The measure of hCG concentration (**table 4.1B**) in trophoblast media at T24, T48 and T72 reflects the occurred syncitialization process.

	Culture Media	24 h	48 h	72 h
[hCG]	1,09	8,27	173,25	721,05
[free β-HCG]	0,28	0,31	0,76	1,78

Table 4.1B: hCG and free β-hCG levels in trophoblast hypoxic cultures at different timing. [hCG] as mUI/mL.

This evidence is confirmed in the graphs below (**figure 4.1.1**) where the free β-hCG secretion in the culture media is higher in timing, meaning the increse of trophoblast syncitialization.

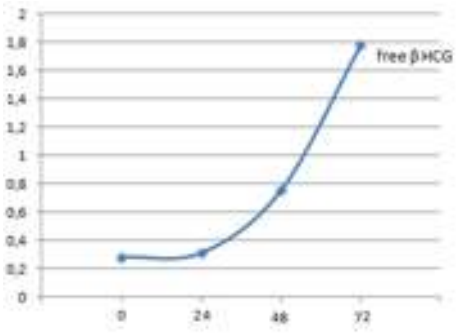
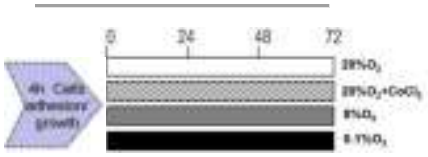


Figure.4.1.1: Increasing time-concentration of free β-hCG in the trophoblast culture media

Mt DNA Content under Hypoxia: Preliminary Data



Preliminary results of the mt DNA content of *in vitro* trophoblast culture model show a trend towards higher mtDNA levels in low O2 conditions. The mtDNA levels appear increased, though not significantly, in both 8% and at 0.1% O2 *versus* 20% O2 at 24, 48 and 72 h (**figure 4.1.2**). mt DNA content in 20%O2+CoCl2 trophoblast cells appears similar to 0.1 O2 ones at all the different culture timing- data not shown (**T24→ 20%:** 356±160 - **8%:** 513±238 - **0.1%:** 509±110; **T48→ 20%:** 236±99 - **8%:** 433±295 - **0.1%:** 458±299; **T72→ 20%:** 259±230 - **8%:** 330±257 - **0.1%:** 525±183). However, cells cultured at low O2 concentrations, and particularly 0.1% O2 at 72 h of incubation, are very low in number, leading to relatively high standard deviations among experiments. Thus, some improvements in the experimental procedure are needed.

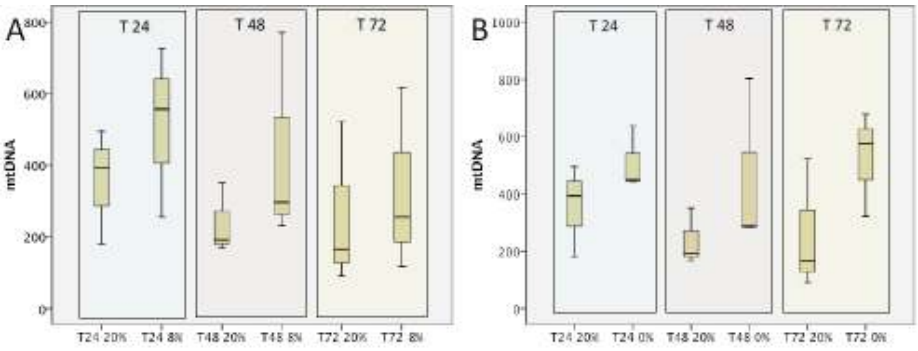


Figure.4.1.2: mtDNA levels in cytotrophoblast cells at A) 20% and 8% O_2 or B) 0.1% O_2 after 24, 48 and 72 h of culture.

4.2 Placental Mesenchymal Stromal Cells (p-MSCs) of normal and IUGR placentas

Clinical Data of the Study Population

Data are presented in **table 4.2**. No significant differences were found between groups for maternal age and pre-pregnancy BMI. As expected, gestational age, placental and fetal weights are significantly lower in IUGR compared to physiological pregnancies.

	Maternal Age (yr)	Pre-Pregnancy BMI (kg/m²)	Gestational Age (weeks)	Placental Weight (g)	Fetal Weight (g)
Term Controls (n = 5)	39.4 ± 2.9	22.2 ± 2.2	39.1 ± 0.0	467.5 ± 120.4	3269.4 ± 345.1
IUGR (n = 6)	37.5 ± 5.8	22.9 ± 5.4	35.0 ± 1.7 **	283.7 ± 59.8 *	1766.5 ± 430.4 **

Table 4.2: Maternal and fetal data of physiological pregnancies and IUGR cases. Data showed as average ± standard deviation. *p<0.05, **p<0.01.

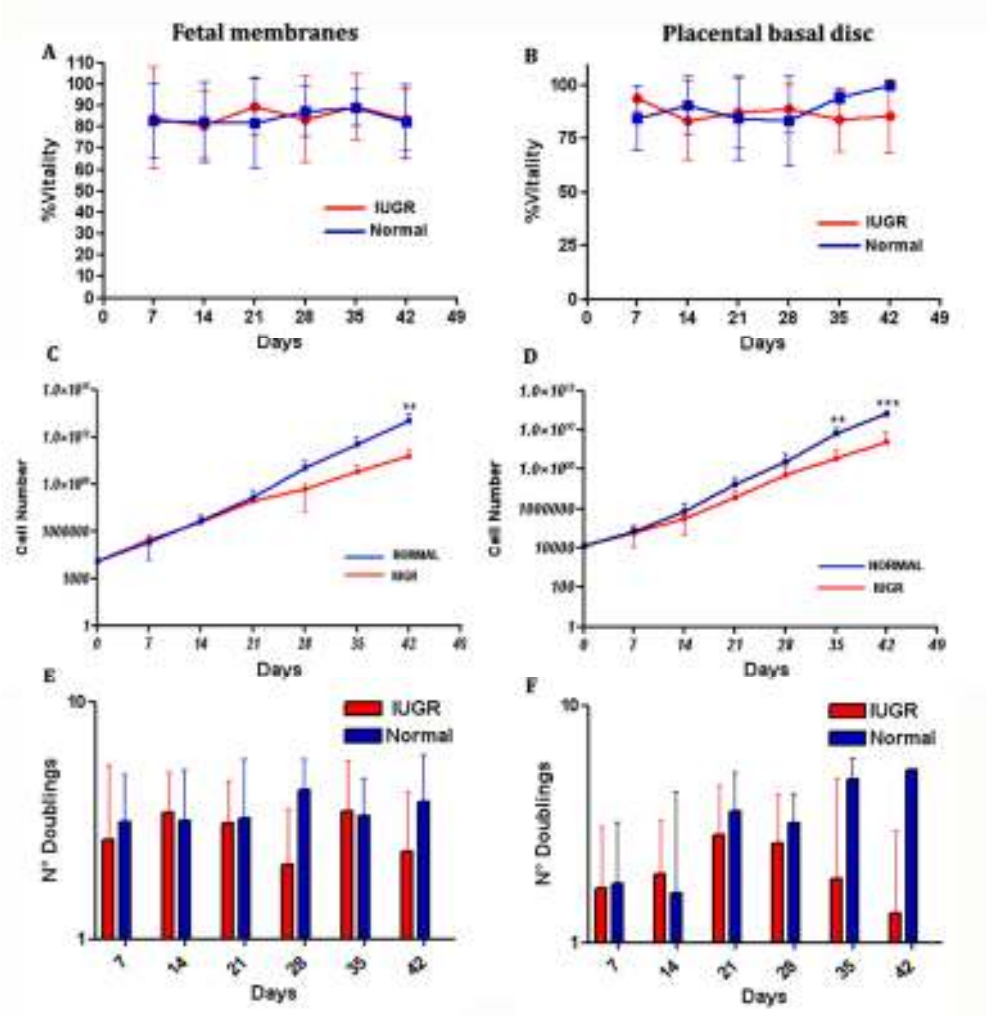


Figure 4.2.1: A and B: Viability of cells isolated from normal and IUGR placentas over a 6-week culture in proliferation medium. Values were obtained by visual counting after Trypan Blue staining. Data are shown as average (± standard deviation in %) of different samples, each performed in three replicates. C and D: Expansion of cells isolated from normal and IUGR placentas observed during 6 weeks of culture. Data are shown as average (± standard deviation in %) of different samples, each performed in three replicates. **p<0.01; ***p<0.001. E and F: Measurements of doubling rate during the culture period, based on visual count.

Viability, Proliferation and Characterization of p-MSCs

Cells were isolated from fetal membranes and placental basal disc and cultured in expansion medium. An high cell viability is reported over 6 weeks for all the analyzed samples, confirming appropriate culture conditions (**Figures 4.2.1 A and B**). IUGR cells display a lower proliferation rate compared to controls after 35 (basal disc) and 42 (fetal membranes and basal disc) days of culture (**Figures 4.2.1 C and D**). Doubling analysis confirm the lower proliferation rate of IUGR basal disc cells after 35 days of culture (**Figure 4.2.1 F**). Flow cytometry analysis attest that, 24 hours after isolation, all samples present an heterogeneous population composed by different cell types (**Figures 4.2.2 A, B, H and I**). After 7 and 30 days of culture we observe a progressive reduction of hematopoietic markers with an increase in mesenchymal markers (**Figures 4.2.2 C, D, F, G, L, M, O** and **P**), indicating an enrichment in cells with a mesenchymal phenotype. This enrichment occurred earlier in IUGR than in AGA samples: at day 7 mesenchymal markers are 1.6-4 fold (fetal membranes) and 2.3-5.8 fold (placental basal disc) higher in IUGR compared to controls. Differences turn out to be statistically significant for CD105, CD44, CD73, CD90 ($p<0.05$) and CD29 ($p<0.001$) in fetal membranes (**Figure 4.2.2 E**) and only for CD29 ($p<0.05$) in placental basal disc (**Figure 4.2.2 N**). After 30 days of culture the cells were characterized for the expression of typical MSC markers by flow cytometry and all resulted positive for CD44, CD105, CD29, CD90 and CD73, while they are negative for CD34, CD133, CD146, CD31 and CD45 thus showing proper mesenchymal stromal cell (MSC) phenotype.

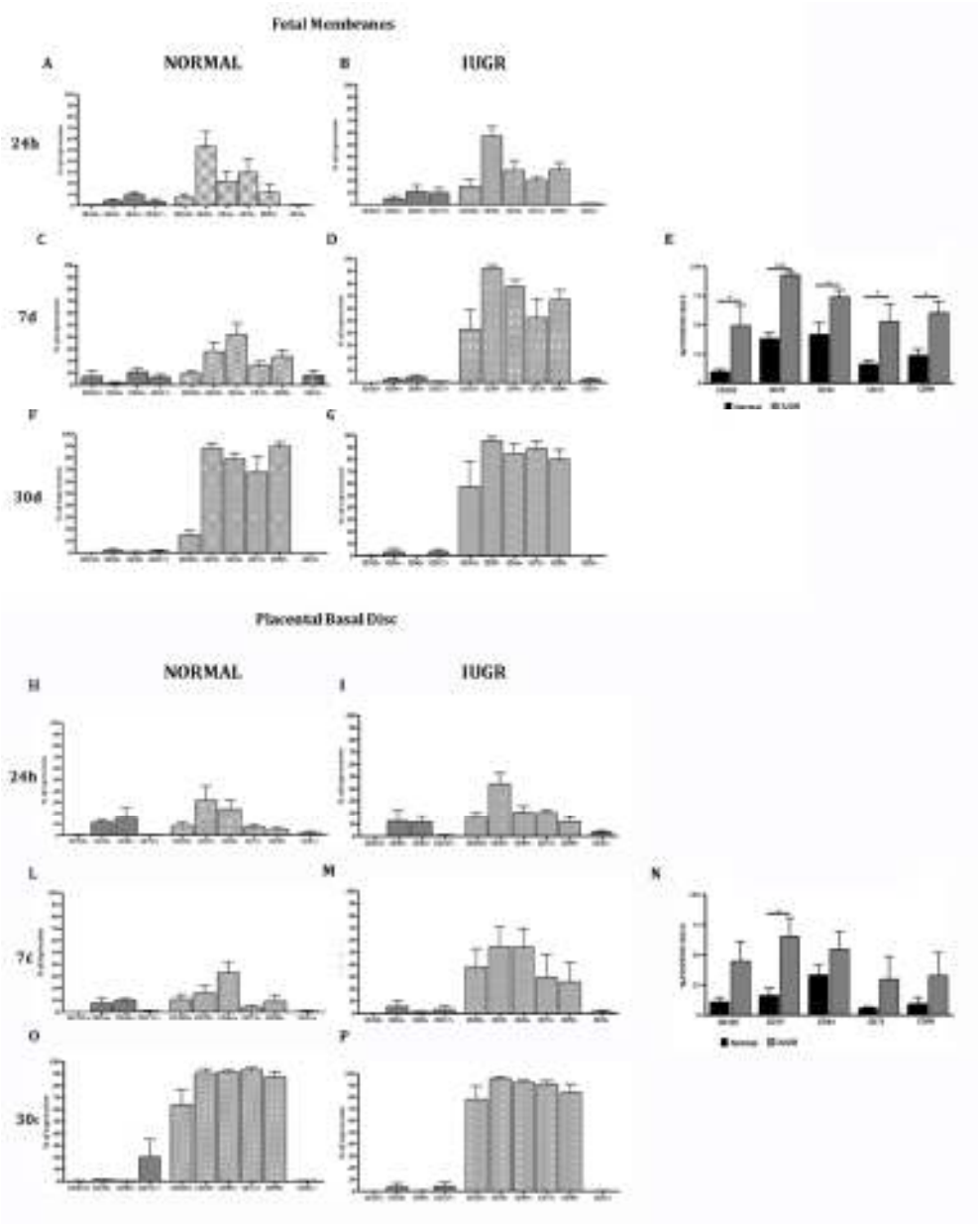


Figure 4.2.2: FACScan immunophenotyping of placental mesenchymal stromal cells (p-MSCs) isolated from normal and IUGR pregnancies cultured in proliferation medium. Hematopoietic, mesenchymal and endothelial markers expression has been analyzed 24 hours after isolation (2A, 2B, 2H, 2I) and after 7 (2C, 2D, 2L, 2M) and 30 days (2F, 2G, 2O, 2P) of culture. At day 7 mesenchymal markers were 1.6-4 fold (fetal membranes) and 2.3-5.8 fold (placental basal disc) higher in IUGR compared to controls. Differences turned out to be statistically significant for CD105, CD44, CD73, CD90 ($p<0.05$) and CD29 ($p<0.001$) in fetal membranes (2E) and only for CD29 ($p<0.05$) in placental basal disc (2N). *$p<0.05$; ***$p<0.001$. After 30 days all samples showed a typical homogeneous immunophenotype of mesenchymal stromal cells (p-MSCs).

Differentiation capacity of p-MSCs

We sought to characterize the differentiation capacity of p-MSCs isolated from normal and IUGR placentas.

Endothelial differentiation

Under endothelial growth medium, Factor VIII-positive cells are quantitatively lower, though not significantly, in cells differentiated from IUGR p-MSCs compared to controls (**Figure 4.2.3**). When 50 ng/ml VEGF is added to the Matrigel culture medium for 3D endothelial differentiation, we observe lower capacity to form endothelial tubular structures in IUGR than in control p-MSCs. Indeed, after 24 hours, no tubular structure formation are observed in IUGR p-MSCs. Few tubular structures are found after 48 and 72 hours in IUGR cultures, although their formation rate remained significantly lower than that in normal p-MSCs. In addition, only normal p-MSCs showed colony-forming capacity (**Figure 4.2.4**).

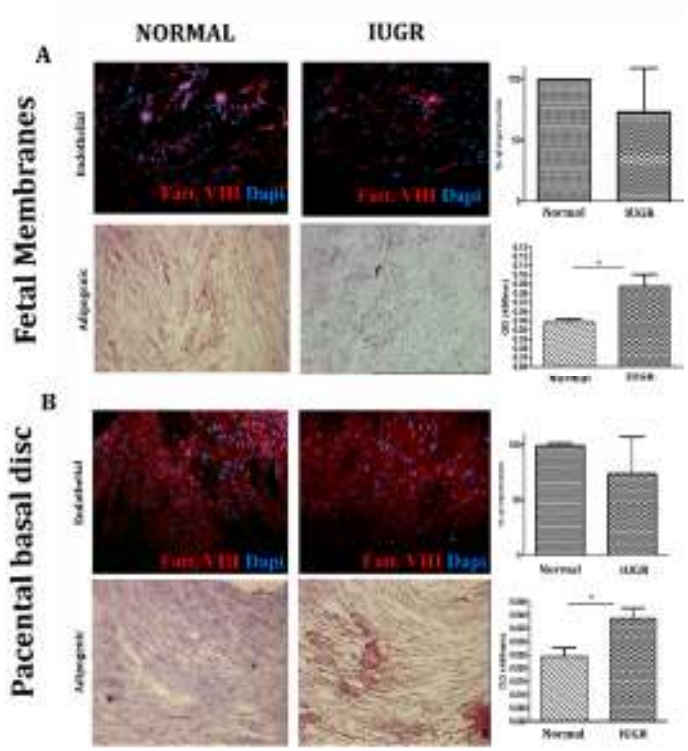
Adipogenic differentiation

Under adipogenic medium, IUGR p-MSCs differentiate 1.7-fold more than normal p-MSCs, as showed by Oil Red O staining (**Figure 4.2.3**). Concordantly, expression of FABP4 and Perilipin A, evaluated by immunofluorescence, is greater in IUGR than in normal-derived p-MSCs (data not shown). Collectively, these *in vitro* findings demonstrate that multipotency of IUGR-derived p-MSCs is altered since their capacity for adipocyte differentiation is increased, whereas their differentiation ability towards endothelial lineage is decreased.

mRNA Expression of Mitochondria-Related Genes

In order to verify a metabolic shift occuring in p-MSCs, we evaluated the mitochondria content of p-MSCs isolated from physiological and IUGR placentas, thus investigating whether mitochondria might be involved in regulating p-MSC cell metabolism. We therefore measured the expression levels of respiratory chain genes *UQCRC1* and *COX4I1* and of the mitochondrial biogenesis activator *NRF1* in 3 IUGR and 3 control p-MSCs.

IUGR compared to normal p-MSCs display a trend towards higher levels of all analyzed genes in the basal disc, and of *COX4I1* in placental membranes, although they do not reach statistical significance (**Figure 4.2.5**).



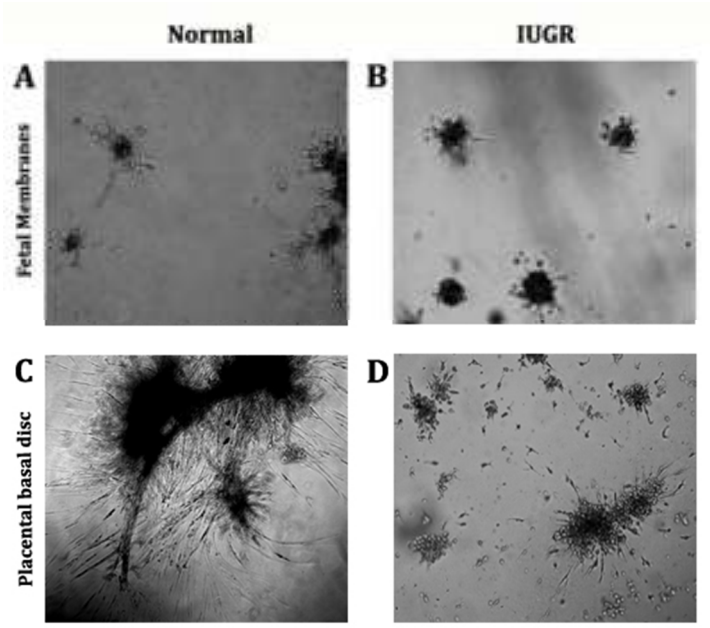
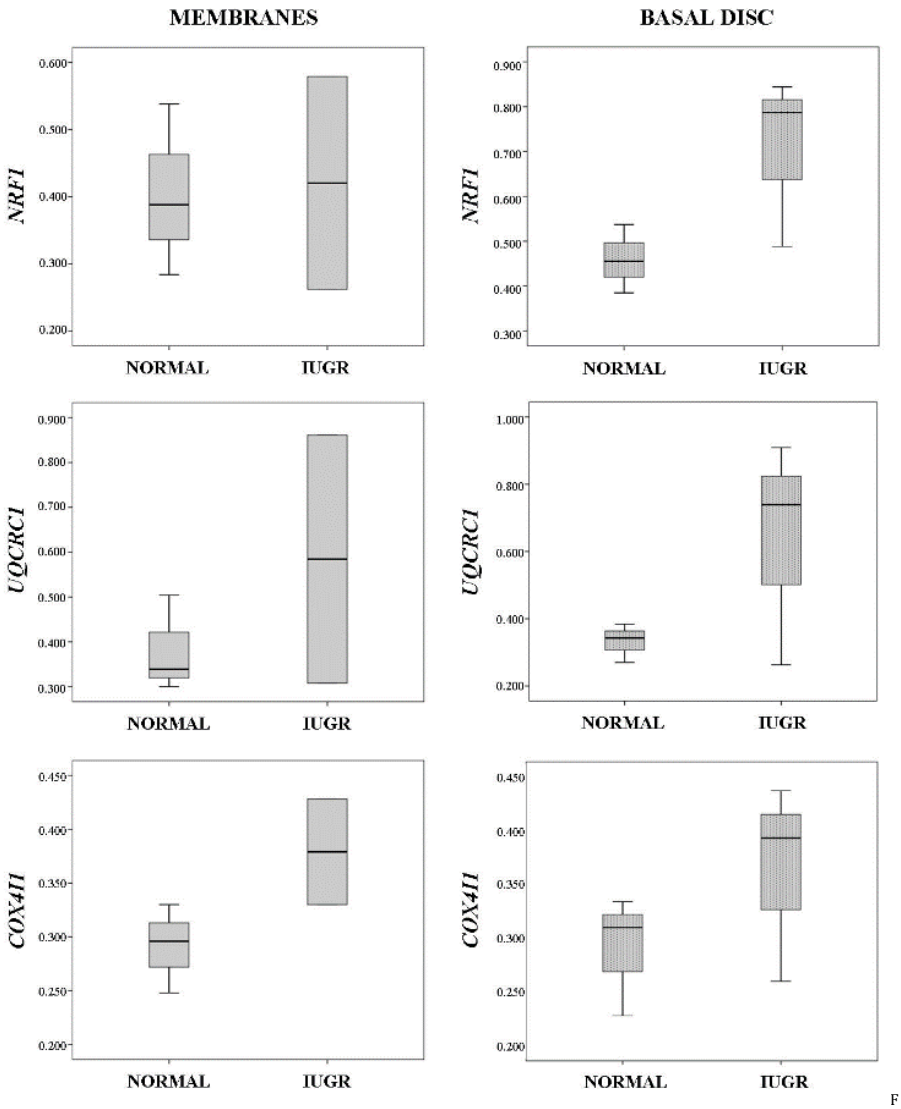


Figure 4.2.3, left: p-MSCs Endothelial and adipogenic differentiation isolated from fetal membranes (A) and placental basal disc (B) in normal and IUGR pregnancies. Differentiation into endothelial and adipogenic lineages: assessed by Factor VIII immunofluorescence and Oil Red O stainings respectively. Nuclei stained blue with DAPI and hematoxylin. Quantitative endothelial differentiation by counting Factor VIII-positive cells x microscope field; adipogenic differentiation by Oil Red O levels in spectrofluorimetry. *p<0.05. Figure 4.2.4, right: Phase-contrast endothelial differentiated p-MSCs morphology isolated from fetal membranes (A, B) and placental basal disc (C, D) in 3D. After 72 hours, p-MSCs appeared as endothelial colonies. Only p-MSCs isolated from normal tissues showed tube-like structures. Magnification 10X.



F

Figure 4.2.5: Gene expression of NRF1 (mitochondrial biogenesis activator), UQCRC1 and COX4I1 (mitochondrial respiratory chain subunits) in p-MSCs isolated from placental membranes and basal disc in normal and IUGR pregnancies. Relative expression values are shown as box plots, indicating the median and the 25th and 75th percentiles.

6 Discussion

Intrauterine Growth Restriction (IUGR), together with preeclampsia (PE), a pregnancy pathology characterized by placental insufficiency, with a multifactorial etiology that still needs to be completely clarified. IUGR is often associated with preterm birth (PTB) [Yu, Rosemberg and Stueve]. The World Health Organization has recently confirmed that PTB is the major cause of neonatal mortality and morbidity, accounting for more than 50% of the early neonatal deaths [WHO, 2005]. Importantly, defects during the intrauterine development may also increase the risk to develop cardiovascular and metabolic pathologies in the adulthood as hypothesized by the "Fetal Programming Theory" [Painter et al., 2008; Barker, 2007; De Rooij et al., 2007], thus becoming a relevant issue also for the general health of future adults. A deeper knowledge of the alterations occurring in IUGR pregnancies has therefore become essential to develop therapeutic tools to prevent fetal, neonatal and future adult complications.

A specific placental phenotype has been associated with IUGR [Cetin and Alvino, 2009], characterized by placentation defects, altered transport of oxygen and nutrients to the fetus [Cetin *et al.,* 2013; Mandò *et al.*, 2013; Mandò *et al.*, 2011; Pardi *et al.*, 1993] and impaired mitochondrial content [Colleoni *et al.*, 2010, Lattuada *et al.*, 2008]. Moreover, the high rate of oxidative metabolism of the active placenta (especially during the 2^-3^ trimesters) is associated with oxidative and nitrative stress, which has been shown to be intensified in pathological conditions, including IUGR [Myatt *et al.*, 2010; Webster *et al.*, 2008].

My purpose is to study, by *ex vivo* experiments and *in vitro* models, different types of placental cells to deeper characterize the placental insufficiency features of IUGR, with specific attention to the consequences of its hypoxic environment.

Characterization of mitochondrial content in IUGR placental tissue and cytotrophoblast cells

In the study population mitochondrial DNA content in IUGR placentas are higher compared to controls. (**figure 1.1**) This evidence, in agreement with previously reported data (Colleoni *et al.,* 2010), complies with NRF1 gene expression levels, that are increased, though not significantly, in IUGR placentas compared to controls. Opposite to what observed in the whole placental tissue, both mt DNA and NRF1 gene expression levels are significantly lower in cytotrophoblast cells isolated from IUGR compared to control placentas, accounting for a decreased mitochondrial content in these cells (**figures 1.1 and 1.2**).

The lower mt DNA content exhibited by cytotrophoblast cells could represent an evidence of the envolvment of further placental cell types (e.g. syncytiotrophoblast, endothelial vascular cells and mesenchymal stromal cells) that may be responsible for the mt content increase in the

whole placental tissue. Indeed, regulation of mitochondrial biogenesis and function by O_2 concentration has been previously observed in other animal tissues (19,60). Changes in mitochondrial content in different types of placental cells may thus possibly be due to the increasing transplacental pO_2 gradient between uterine and umbilical venous blood, previously described in IUGR pregnancies (39) and also reported in IUGR animal models (49). The increased maternal-fetal O_2 gradient (40,39) may in fact expose endothelial and trophoblast cells to two extremes of O_2 environment with opposite changes in mitochondrial biogenesis. Endothelial cells covering the blood vessel walls might be the most affected by O_2 variations. We could speculate that the higher mitochondrial activity observed in trophoblast cells of IUGR fetuses might cause additional fetal vascular damage via an excess of reactive oxygen species (ROS). Such damage could eventually trigger higher mitochondrial biogenesis in fetal and placental endothelial cells.

The difference between mtDNA content of cytotrophoblast cells and total placental tissue in IUGR fetuses might also be due to differential exposure to progesterone. Progesterone and other steroids, such as estrogens later described, regulate mitochondrial biogenesis and might play a role in the pathogenesis of IUGR in humans. Partial progesterone withdrawal in rats induces placental and fetal growth restriction (34). Thus, progesterone production, which occurs in the syncytiotrophoblast, but not in the cytotrophoblast cell, may lead to different mitochondrial levels in these different cell lineages of the IUGR placenta.

Mitochondrial function in IUGR placental cytotrophoblast cells

In cytotrophoblast cells isolated from IUGR placentas, mRNA expression of the RCC subunits CII, CIII and CIV shows a trend towards lower levels compared to controls, though not significantly when the Bonferroni adjustment is applied to *t*-tests. Their protein expression do not present any difference between IUGR and control cells. (**see figure 2.1**).

In contrast, mt bioenergetics efficiency represented by cytotrophoblast cells O_2 consumption is higher in IUGR *versus* controls. Thus, despite the protein content of RCC subunits is not altered, their activity is significantly increased in IUGR cytotrophoblast cells. This may be due to a defective RCC assembly in supercomplexes. Supercomplex assembling, a post-translational modification required for RCC stability and functionality, allows more efficient electron flux and higher availability of substrates (27,56,23). A possible more efficient supercomplex assembly may explain the increase in respiratory capacity of IUGR cells.

Although the mitochondrial content in cytotrophoblast cells of the IUGR group is significantly lower than in controls, these same mitochondria were able to sustain higher total cellular

respiration rate. In addition, when all samples are analyzed together, O_2 consumption is inversely correlated both with mtDNA content of cytotrophoblast cells and with fetal and placental weights (**figure 2.4**). These findings altogether might suggest a functional compensatory effect to the decreased mitochondrial content, the more the growth restriction the higher the mitochondrial O_2 consumption.

Another remarkable observation derives from the analysis of data according to umbilical artery Doppler velocimetry (PIumb). IUGR cases have been previously associated with cerebral metabolic and maturation changes driven by hypoxia, as shown by in utero MR spectroscopy (8) and post-natal MR imaging (47). In our population O_2 consumption presents a significantly higher increase compared to controls in IUGR with abnormal PIumb, rather than in IUGR fetuses with normal PIumb. This underlines the role of umbilical artery Doppler velocimetry as a marker of severity in IUGR.

These data suggest that possible compensatory mitochondrial mechanisms occur in IUGR to sustain fetal growth under conditions of severe placental vascular insufficiency. Further studies are needed to elucidate the consequences of these findings, especially as regards possible enhanced ROS generation and in different phases of placental development.

Regnault *et al.* (49) suggest that in IUGR the higher transplacental pO_2 gradient and the lower umbilical vein pO_2 are the result of a lower ratio between low and high hindrance sites in the placental epithelium. The higher rate of O_2 consumption found in cytotrophoblast cells of IUGR human fetuses can contribute to generate high hindrance sites in the placenta, together with possible local decreases in the IUGR transplacental diffusion distance. This possibility has been demonstrated to date in a mouse model of IUGR (14).

Thus, our data suggest that altered O_2 delivery to IUGR fetuses might also be due to increased O_2 consumption within trophoblast cells, possibly representing one of the key factors leading to growth restriction. However, we cannot exclude that placental changes may be the result of a reduced fetal O_2 consumption due to the slower rate of growth and thus a decreased need for oxygen. We indeed have previously reported a significant reduction of fetal O_2 consumtion even on a per kilogram basis in IUGR (46).

The changes occurring in IUGR might be influenced by several causes, such as Caloric Restriction. Indeed, strong links have been previously described between calorie restriction and changes in the mitochondrial machinery of different tissues (12,30). Growth Restricted placentas usually present many characteristics leading to calorie restriction and mitochondrial defects, such as poor expression and activity of nutrient transporters (7,33,53,10,18), low activity of the

glutamine-glutamate metabolism and poor control of mitochondrial lipid peroxidation (33,50), with increased sensitivity to mitochondrial oxidative stress and ROS production. Moreover, micronutrients imbalance might also be involved in IUGR mitochondrial alterations (32, 9).

Estrogen-Related Receptor (ERRγ) and CYP19 placental expression

Several mechanisms may be implicated in altered mitochondrial function. Estrogen-Related Receptor (*ERRγ*), involved in mitochondrial biogenesis and functions (31,48), regulates estrogen production (21). In placenta, *ERRγ* is able to bind the promoter region of CYP19 aromatase, one of the main enzymes of the estrogen pathway production (in particular 17β-estradiol), enhancing its gene expression [Stocco, 2012]. Thus *ERRγ* possible increase, due to mt biogenesis alterations in IUGR, may also lead, via-CYP19, to higher estradiol levels in IUGR placentas, contributing to abnormal placental vascularization and fetal maturation in IUGR pregnancies (42,44).

To assess this hypothesis, *ERRγ* and *CYP19* gene expression have been investigated for the first time in human IUGR placentas, by measuring their mRNA levels both in whole placental tissue and its isolated cytotrophoblast cells.

In whole placental tissue *CYP19* presents increased expression levels in the IUGR group compared to controls; in particular *CYP19* mRNA levels are progressively higher with IUGR severity. We also find higher *ERRγ* expression in IUGR cases, though not significantly. This represents a further new evidence suggesting altered mitochondrial biogenesis and content in IUGR, thus expanding our newest study on growth-restricted placentas [Mandò *et al.,* 2014]. Indeed, *ERRγ* shows the same expression pattern of NRF-1, described above, giving a further evidence of its role as an active transcriptional factor in placental mt biogenesis. This suggests a potential role for ERRγ in the impaired IUGR metabolism. Evidences on animal models support the hypothesis that increased *ERRγ* expression in IUGR might be a restore attempt by increasing mt biogenesis via *ERRγ* overexpression [Roberts *et al.,* 2001]. Mayeur and colleagues showed that rats growth-restricted placentas, induced by maternal undernutrition, present impaired mitochondrial function [Mayeur *et al.,* 2013]. On the other hand, an active ERR regulatory pathway seems to be required in lung mitochondria adaptation to high-altitude environment in a mice model [Chitra and Boopathy, 2014]. Likewise, mice lacking one copy of ERRγ exhibit a decreased muscle mt functionality and exercise capacity.

The role of ERRγ in growth restriction pathogenesis could also be exerted through its interaction with CYP19 aromatase as previously said. Its higher levels in IUGR placentas compared to controls may be actually due to the action of ERRγ on CYP19 gene promoter.

Several *in vitro* studies in other tissues [Liu *et al.,* 2010; Pedram *et al.,* 2006; Stirone *et al.,* 2005] suggest an estrogen protective role against oxidative stress, particularly for estradiol. This is mediated by the Estrogen Receptor (ER alpha) and leads to a reduction of mitochondrial Reactive Oxygen Species (ROS) levels. Based on our results, we could thus speculate that the higher CYP19 expression, possibly mediated by ERRγ, may be a guided estrogen protective action for the growth-restricted placenta status, also considering that CYP19 expression progressively increases with IUGR severity.

Opposite to their placental tissue expression, *ERRγ* gene expression levels in cytotrophoblast cells isolated from those placental tissues are significantly decreased in the IUGR group compared to controls. Interestingly, previous *in vitro* experiments show that trophoblast cells cultured under hypoxic conditions (2% O_2; 5% CO_2) present an O_2-dependent *ERRγ* gene expression with lower levels due to insufficient oxygen supply [Kumar and Mendelson 2011]. Among different placental cell types, cytotrophoblast cells have been choosen since they are very important for placental metabolism and nutrient transport, together with multinucleated syncytiotrophoblasts. However, as well as for mt DNA content, other cell types (i.e. endothelial cells or placental stromal cells) could be responsible for the increase of *ERRγ* expression in the whole placental tissue. Moreover, as already mentioned, oxygen concentration (which presents an altered gradient within IUGR placentas) regulates mt biogenesis through its transcriptional activators, with various effects depending on the different histological region [Leduc *et al.,* 2010; Zung *et al.,* 2007].

CYP19 gene expression do not show any difference between IUGR and controls in cytotrophoblast cells, though it positively correlates with *ERRγ* levels. It is known that *CYP19* expression increases with cytotrophoblast cells differentiation, up to a maximum production in the syncytiotrophoblast [Kamat and Mendelson, 2001]. Indeed, low *CYP19* levels resulted in cytotrophoblast cells, which might complicate the detection of differences between IUGR and control samples.

Finally, the significant positive correlation linking maternal BMI and the expression of both *ERRγ* and *CYP19* genes in whole placental tissue is worth of interest. On the contrary, in trophoblast cells this correlation shows a negative trend. A recent *in vitro* study on trophoblast cells reveals an estradiol-dependent regulation of leptin production through ER-alpha receptor, whose levels usually increase together with *ERR* expression [Gambino *et al.,* 2012]. Moreover, evidences from ER alpha or CYP19 knock-out in mice suggest that ERR could cooperate in preventing an adipose phenotype [Heine *et al.,* 2000, Jones *et al.,* 2000]. Leptin is known to increase during pregnancy [Cetin *et al.,* 2001, Widjaja *et al.,* 2000] and to function as an anti-

obesity hormone synthetized in placenta by both cyto and syncytiotrophoblast cells [Poidatz *et al.,* 2014; Maymo *et al.,* 2011]. The measure of plasmatic levels of both leptin and 17β estradiol in maternal blood is needed to assess this hypothesis.

In vitro experiments to explore possible mechanisms of mitochondrial alterations in IUGR placental cells

Fluctuations in placental oxygen concentration may generate oxidative stress (OxS). Indeed, Intrauterine Growth Restriction presents altered placental and fetal oxygenation [Pardi *et al.*, 1993, Cetin and Alvino, 2009], together with vascular defects and chronic low-grade inflammation leading to increased oxidative stress [Menon, 2014]. As mitochondria are the major producers of intracellular reactive oxygen (O_2) species through free radicals generated by the mt oxidative phosphorylation, altered intrauterine O_2 conditions might affect mt DNA content and function, leading to increased oxidative stress in IUGR placental cells [5]. My purpose is to reproduce in trophoblast primary cell lines, different O_2 conditions that placentas may be exposed to during a pathological pregnancy. Exposure of trophoblast cultures to hypoxia is an *in vitro* model commonly used in the last few years to mimic pregnancy oxygenation normal and pathologic environments [Williams *et al.*, 2012; Oh *et al.,* 2011; Tuuli *et al.*, 2011; Baumann *et al.,* 2007; Nelson *et al.,* 2003; King *et al.*, 2000]. Here I reported preliminary data showing that the oxygen lack in cytotrophoblast cells leads to increased mt DNA levels. The evidence that O_2 levels may regulate mt biogenesis in cytotrophoblast cells highlights their deep sensitivity to O_2 conditions. However, further data are needed to confirm these preliminary results. Although all attempts in adapting the model to each study purpose, hypoxic coltures remain an open challenge. In fact, primary cytotrophoblast cultures are very sensitive to O_2 concentration, so that even 20% O_2 is deleterious for growth conditions. Moreover, hypoxia (0.1 - 1% O_2) produces metabolic footprints in the conditioned culture medium that might affect trophoblast metabolomic responses [Zamudio/Tuuli]. Among problems affecting the hypoxic *in vitro* model, there is the high cell mortality, especially under 0.1% O_2 condition: in next experiments, less strong hypoxic condition (1.5% O_2) will be tested to enhance trophoblast cells vitality. It will also be important to choose the best timing of culture evaluating the effects of hypoxia levels and durations on gene expression patterns of human trophoblasts. For instance, it is my intent to extend the initial adhesion time from 4 to 12 hours: this will allow a trophoblast better adhesion to the plate, hopefully stabilizing the number of plated cells enhancing the amount and quality of the derived mt DNA. Moreover, future experiments will reproduce hypoxia/re-oxygenation intervals characterizing placental insufficiency and

generating OxS. I will measure levels of apoptosis and autophagy markers (e.g. TNF-α, p53, caspases), that are consequences of stress on placental trophoblast cells.

Placental - Mesenchymal Stromal Cells in IUGR and control placentas

I then shift my investigation on a different placental cell type: placental - Mesenchymal Stromal Cells (p-MSCs)

In vitro experiments have been performed to isolate and characterized p-MSCs from human placentas affected by Intrauterine Growth Restriction and from healthy human placentas. p-MSCs have never been investigated before in IUGR pregnancies. However, their role might be crucial in placental insufficiency pathologies. Recent studies on preeclamptic pregnancies report that PE p-MSCs show pro-inflammatory and anti-angiogenic features, that may result in abnormal placental development [Rolfo 2013].

In the performed experiments, mesenchymal markers enrichment during p-MSCs cultures and multipotent differentiation abilities confirm the successful isolation and selection of a mesenchymal stromal cell population from placental membranes and basal disc of both physiological and IUGR placentas. As attested by flow cytometry data, the p-MSC population is earlier selected in IUGR placentas. Thus, the faster selection of the mesenchymal cell type in IUGR cultures might represent a compensatory mechanism to metabolic alterations occurring in IUGR placental cells and/or to the adverse IUGR placental environment. The lower proliferation rate characterizing IUGR pMSCs (especially after 35 days of culture) has been previously reported in PE with fetal and/or placental compromise compared to physiological pregnancies [Rolfo, 2013]. During placentation process, this reduction in p-MSCs proliferation rate could impair the primary villi formation and consequently trophoblast development, since MSCs both serve as structural support and exert a paracrine activity on trophoblast cells.

Moreover, IUGR p-MSCs population display lower endothelial and higher adipogenic differentiation potentials compared to controls. During pregnancy, pMSCs usually contribute to both vasculogenesis and angiogenesis [Arroyo 2008; Gourvas 2012; James 2014] through the endothelial progenitor's capacity of tissue regeneration also by induction of new vessels, therefore increasing tissue perfusion and oxygenation [Demir 2007; Charnock-Jones 2004]. Interestingly, several studies report some alterations in maternal and fetal endothelial progenitor or in the angiogenic capacity of IUGR placental cells [Calcaterra 2013; Sipos 2013; Riddell 2013]. The mechanisms behind these p-MSCs alterations in IUGR still need to be clarified and may be associated with their reduced capacity to differentiate in endothelial cells. Opposite to endothelial differentiation ability, the adipogenic potential in pMSCs from IUGR is

increased compared to controls. IUGR newborns present increased risk of adult obesity and metabolic syndrome [Simmons 2008, Gluckman 2005]. In IUGR animal models, several alterations in both adipose tissue structure and endocrine system have been demonstrated. As these changes are evident early in life, the predisposition to obesity may be programmed in utero [Ross 2013].

Mithocondrial (mt) DNA, together with mRNA levels of mt biogenesis transcription factors (e.g. NRF1 and 2 and ERRγ) and of mt Respiratory Chain Complexes (RCC), are well accepted biochemical markers to assess mitochondria content. Thus, to further characterize IUGR pMSCs, their mitochondrial (mt) content is investigated by measuring gene expression levels of the mt biogenesis activator *NRF1* and of the Respiratory Chain genes *UQCRC1* and *COX4I1* (two subunits respectively belonging to the RC CIII and CIV). Mesenchymal stem cell metabolism is known to be mainly anaerobic, with a shift towards an aerobic mitochondrial metabolism reported during differentiation, when increase of mt biogenesis, oxygen consumption and ATP production occur [Chen 2008; Hofmann 2012]. Interestingly, p-MSCs cultured with no differentiating medium present a trend towards higher *NRF1, UQCRC1* and C*OX4I1* expression levels in IUGR basal disc samples compared to controls and higher *COX4I1* levels in IUGR placental membranes; these differences are not statistically significant likely because of the low sample number. Nevertheless, they might account for metabolic alterations in IUGR p-MSCs, showing a possible shift to aerobic metabolism, with the loss of the metabolic characteristics that are typical of multipotent and undifferentiated cells. These results are consistent with recent data showing a higher mitochondrial content in IUGR placental tissue [Lattuada, 2008].

Limitations

Placental O_2 consumtion is a complex feature resulting from the activity of different cell lineages. O_2 consumtion data are obtained only from cytotrophoblast cells due to the protocol needed to isolate a proper amount of single cells from the entire placental tissue. Thus, we can only speculate about the possible contribution of cytotrophoblast cells to the total placental O_2 consumtion, and not exclude that different cell types may give different results.

Another possible limit is the different gestational age between cases and controls. This is a limit of all studies investigating human IUGR compared with control-term placentas. However, to the best of our possible experimental design, we do not observe any significant negative correlation between gestational age and the O_2 consumption of CIV, which presents the highest significant difference between IUGR and controls among respiratory chain complexes.

Gestational age correlates to fetal weights but may independently alter mitochondrial number and/or function because of differences in hormone secretion and potential changes in placental O2 consumption as gestational age advances. No studies have addressed the influence of gestational age on placental or trophoblast cells O2 consumption in humans.

The different gestational age between IUGR and controls may rappresent again a possible limit of the analyses on ERRγ and CYP19 gene expression. However, both gene expression are not related to gestational age in our population, except for *CYP19* mRNA levels in the placental tissue. However, though all IUGR cases with both normal and abnormal PI have significantly lower gestational age compared to controls, only the most severe IUGR show significantly higher levels of *CYP19* expression, thus suggesting that gestational age is not involved in the reported differences between cases and controls. Moreover, when comparing gestational ages of IUGR with normal PI and cases with abnormal PI, we do not find any significant difference (t-test: p=0.205, data not shown).
CYP19 gene expression have been analyzed assuming that it may represent an index of aromatase content in placental tissue. However, post-translational modifications (glycosylation and phosphorylation) may occur, affecting its functional activity. Further experiments will be planned to asses this aspect.

A potential limitation of the study on placental mesenchymal stromal cells (p-MSCs) is represented again by the different gestational age of the IUGR compared to the control group, previously discussed. Another important observation regard the analysis on IUGR placentas is that has been performed at delivery, whereas placental abnormal development of IUGR pathology is supposed to start already at the beginning of placentation. Therefore, the observed features might also be the consequence of an altered placental environment influencing pMSC.

Conclusion

Taken together, reported data highlight mitochondrial alterations occurring in placentas of Intrauterine Growth Restricted pregnancies, through *ex vivo* and *in vitro* approaches.
These results shed genuine new data into the complex physiology of placental oxygenation in IUGR fetuses. Mitochondrial content is higher in IUGR total placental tissue compared with normal pregnancies at term. This difference is reversed in cytotrophoblast cells of IUGR fetuses, which instead present higher mitochondrial functionality. These findings suggest different mitochondrial features depending on the placental cell lineage.

Indeed, our results on placental Mesenchymal Stromal Cells, showed higher levels of genes accounting for mitohcondrial content and function.

The increased placental O_2 consumption by placental tissue may represent a limiting step in fetal growth restriction, preventing adequate O_2 delivery to the fetus. This limitation has potential consequences on fetal O_2 consumption both in animal models and in human IUGR.

7 Bibliography

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