Epigenetic modifications associated with intrauterine growth restriction

Carla Sofia Caniçais Rodrigues
Mestrado em Biologia Celular e Molecular
Departamento de Biologia
2017

Orientador
Sofia Dória Príncipe dos Santos Cerveira, Professor Auxiliar, FMUP

Coorientador(s)
Cristina Joana Moreira Marques, Investigador Auxiliar (Investigador FCT), FMUP

Carla Maria Almeida Ramalho, MD, Professor Auxiliar Convidado, FMUP

U.PORTO

FMUP FACULDADE DE MEDICINA UNIVERSIDADE DO PORTO

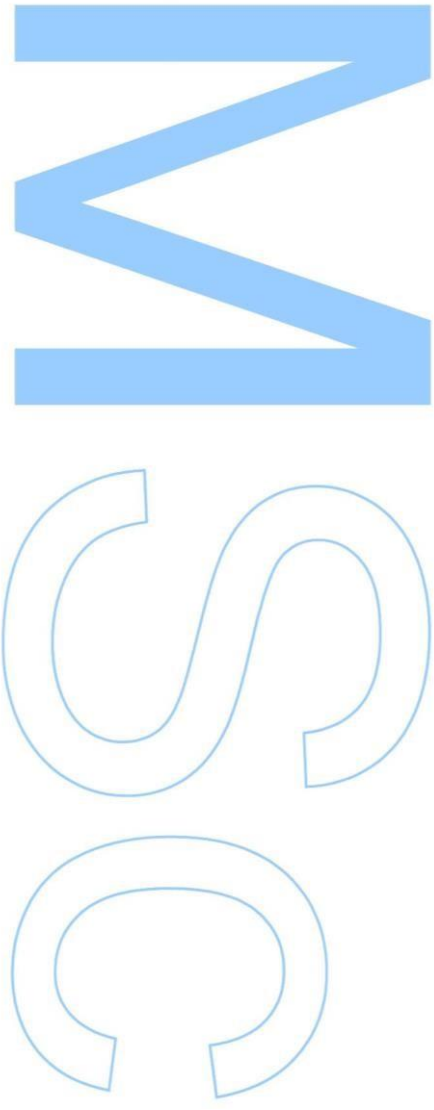
U.PORTO

FC FACULDADE DE CIÊNCIAS UNIVERSIDADE DO PORTO

Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, _____/_____/_____



Autor

Carla Sofia Caniçais Rodrigues

Rua do Campo Alegre, 4169-007 Porto, PORTUGAL, up201502145@fc.up.pt, 22 040 2000

Orientador

Sofia Dória Príncipe dos Santos Cerveira, Professor Auxiliar, FMUP

Al. Prof. Hernâni Monteiro, 4200 - 319 Porto, PORTUGAL, sdoria@med.up.pt, 22 551 3647.

Coorientador

Cristina Joana Moreira Marques, Investigador Auxiliar (Investigador FCT), FMUP

Al. Prof. Hernâni Monteiro, 4200 - 319 Porto, PORTUGAL, cmarques@med.up.pt, 22 551 3647.

Esta dissertação incluiu ainda um Coorientador Clínico que foi responsável pela informação clínica, contacto com as gestantes e seleção das amostras.
Carla Maria Almeida Ramalho, MD, Professor Auxiliar Convidado, FMUP
Al. Prof. Hernâni Monteiro, 4200 - 319 Porto, PORTUGAL, carlaramalho@med.up.pt, 22 551 3647.

Eu, Carla Sofia Caniçais Rodrigues, aluna com o número 201502145 do Mestrado de Biologia Celular e Molecular da edição de 2016/2017, declaro sob compromisso de honra ser autora do texto apresentado, não sendo este plagiado. Declaro também que tomei conhecimento das consequências que advém de situações de plágio.

Porto, 29 de setembro de 2017

Carla Sofia Caniçais Rodrigues

Agradecimentos

O percurso até finalizar a dissertação foi de intensa aprendizagem científica e pessoal, que só seria possível graças a todas as pessoas que me apoiaram e ajudaram ao longo deste período.

Primeiramente, gostaria de agradecer ao Professor Doutor Alberto Barros, diretor da Unidade de Genética da Faculdade de Medicina da Universidade do Porto, pela oportunidade de realização do trabalho no serviço e por me ter disponibilizado todo o material que utilizei no decorrer da investigação. Obrigada pela simpatia e confiança.

À Prof. Doutora Sofia Dória, orientadora desta dissertação, agradeço pela ajuda e pelo voto de confiança. Também agradeço todos os bons conselhos, as palavras de incentivo, a preocupação e a simpatia que transmitiu no decorrer da tese. Muito obrigada!

À Doutora Joana Marques, co-orientadora da dissertação, o meu sincero agradecimento por todos os ensinamentos transmitidos que permitiram o desenvolvimento desta tese. Agradeço a disponibilidade, a atenção dispensada, a preocupação e apoio que sempre me concedeu em momentos de trabalho mais críticos. Muito obrigada por tudo.

À Prof. Doutora Carla Ramalho, agradeço pela recolha das placentas e da informação clínica dos participantes desta dissertação.

Um agradecimento muito especial a todas as pessoas da Unidade de Genética da Faculdade de Medicina da Universidade do Porto, pela partilha dos conhecimentos, pelos bons momentos e por todas as risadas partilhadas. Guardo um bocadinho de cada um comigo. Obrigada por me mostrarem que o trabalho e a boa disposição podem estar sempre de “mãos dadas”.

Às minhas “Itas”, Dianita, Joanita e Sonita, obrigada pelos melhores anos da minha vida. Agradeço-vos por estarem sempre presentes, por todas as aventuras que partilhamos e por me ensinarem o verdadeiro valor da amizade. “O que Aveiro une ninguém separa”.

Às minhas amigas de infância, Bia, Dani e João, obrigada pelos anos e anos de amizade. Agradeço-vos por me mostrarem que as amizades, quando são verdadeiras, podem permanecer durante muitos anos apesar da distância e das diferenças de cada uma. Obrigada por todo o apoio, por todas as risadas e por tudo o que vivemos. Seremos amigas até sermos velhinhas.

Finalmente, à minha família, agradeço imenso pelo apoio e presença em todas as etapas do meu desenvolvimento académico e pessoal. À minha mãe e à minha *abuelita,*

um obrigado não é suficiente por tudo o que me ensinaram e todos os valores que me incutiram. Tudo o que consegui até agora e o que sou a vocês se deve. Muito obrigada por estarem sempre presentes na minha vida e por serem os meus pilares. Ao meu primeiro melhor amigo, o meu irmão, agradeço toda a companhia, os conselhos e as aprendizagens desde sempre. És o melhor irmão que podia desejar e um modelo de pessoa a seguir. Ao meu pai, agradeço por todo o apoio e por todas as palavras de incentivo. Apesar da distância, o apoio, o amor e as palavras reconfortantes nunca faltaram.

Resumo

A Restrição de Crescimento Intrauterino é uma condição multifactorial na qual o feto é incapaz de alcançar o seu crescimento normal. A insuficiência placentária é uma das causas mais comuns e pode ter complicações de curto ou longo prazo, após o nascimento, e em casos mais severos pode levar a um abortamento. A alteração da expressão de genes da placenta está associada com um desenvolvimento placentário anormal, que pode resultar na restrição do crescimento do feto. Vários genes expressos pela placenta são *imprinted* e podem ser controlados em regiões diferencialmente metiladas, pela metilação de DNA. Recentemente, foi descrita uma outra modificação epigenética, a 5 hidroximetilcitosina (5-hmC). Níveis de 5hmC estão associados ao tamanho fetal ao nascimento, contudo, não existem estudos que avaliem esta modificação em casos com RCIU.

O objectivo principal desta investigação foi encontrar biomarcadores que possibilitem a previsão da restrição do crescimento intrauterino. Para realizar o objetivo proposto, a análise da expressão de genes *imprinted* (*CDKN1C, IGF2, KCNQ1, H19, PEG10, MEST* e *PHLDA2)* foi realizada, assim como o estudo da metilação de KvDMR1 e dos níveis globais de hidroximetilação de DNA. Com esse intuito, um PCR quantitativo em tempo real foi executado em vinte e duas amostras de placentas a termo com RCIU e em onze amostras de placentas normais.

A análise dos resultados mostrou alterações significativas na expressão dos genes *CDKN1C*, *PHLDA2* e *PEG10* entre amostras com RCIU e amostras normais, observando-se um aumento da expressão nos casos com restrição de crescimento. A sobrexpressão desses genes *imprinted* é coerente com o descrito na literatura e com a teoria do conflito parental, com a exceção do gene *PEG10.* A sobreexpressão deste gene paternalmente expresso sugere uma resposta compensatória à RCIU. Para além dos genes *imprinted,* genes que codificam enzimas envolvidas na metilação e hidroximetilação estão significativamente sobre-expressos em amostras com RCIU, tal como é o caso de *DNMT1*, *DNMT3A* e *TET3*. Para o estudo dos padrões de metilação de KvDMR1 foram executadas técnicas de modificação bissulfito, clonagem e sequenciação em quatro amostras de RCIU e em três amostras normais. Os resultados finais da sequenciação demonstraram que as amostras de RCIU apresentavam uma tendência para estarem hipermetiladas, contudo a diferença entre os dois grupos não foi significativa. Essa tendência foi corroborada pelo aumento da expressão dos genes que codificam as metiltransferases do DNA em amostras de fetos diagnosticados com RCIU. Para além da ausência de uma diferença significativa entre os dois grupos, observou-se que a associação entre a expressão dos genes,*CDKN1C* e *PHLDA2*, e a

metilação de KvDMR1, apesar de positiva, não era significativa. A hidroximetilação global da totalidade das amostras foi avaliada com um método colorimétrico e foi observada a presença de 5-hidroximetilcitosina nas amostras da placenta. Contudo, os resultados mostraram um mudança não significativa entre os dois grupos (RCIU vs normais).

Em conclusão, os resultados obtidos durante esta tese permitiram a identificação de dois potenciais biomarcadores de RCIU, *CDKN1C* e *PHLDA2*. Para além disso, o estudo confirmou a presença de hidroximetilação na placenta e mostrou que as amostras de fetos com restrição de crescimento detinham uma tendência para a hipermetilação de KvDMR1. A ausência de associação significativa entre a expressão dos genes e a metilação pode ser justificada pela ação de outro mecanismo *trans-acting.* Contudo, a tendência para a hipermetilação de KvDMR1 e o aumento da expressão dos genes *PHLDA2* e *CDKN1C*, em amostras com RCIU, manifesta uma possível associação entre esses parâmetros e a RCIU. Assim, em estudos de metilação ulteriores, o tamanho da amostragem deve de ser superior ao que foi analisado nesta investigação.

Abstract

Intrauterine growth restriction is a multifactorial condition in which the fetus is not able to reach its normal growth potential. The placental insufficiency is one of the most common causes and may have short-term or long-term complications after birth and in severe cases can lead to abortion. The abnormal placental gene expression is associated with an abnormal placental development, resulting in growth restriction of the fetus. Several placental genes are imprinted and may be controlled at Differentially Methylated Regions by DNA methylation. Recently, another epigenetic modification has been described, the 5-hydroxymethylcytosine. 5-hmC levels are associated with fetal size at birth, however, there are no studies evaluating this modification in IUGR cases.

The main aim of this research was to find biomarkers that allow the prediction of intrauterine growth restriction (IUGR). To accomplish the proposed aim, imprinted gene expression (*CDKN1C, IGF2, KCNQ1, H19, PEG10, MEST* and *PHLDA2)* analysis was performed together with the study of DNA methylation in KvDMR1 and global DNA hydroxymethylation levels. In order to do that, quantitative Real-Time PCR was performed in twenty-two term placental samples of fetus diagnosed with IUGR and in eleven placental samples of normal fetus.

The results analysis showed significant changes in *CDKN1C*, *PHLDA2* and *PEG10* expression between IUGR samples and normal samples, observing an increase in expression in IUGR cases. The overexpression of this imprinted genes is consistent with

the previously described in the literature and with parental conflict theory, with the exception of the *PEG10*. The overexpression of this paternally expressed gene suggests a compensatory response to IUGR. In addition to imprinted genes, the expression of genes that code for enzymes involved in methylation and hydroxymethylation were significantly overexpressed in IUGR samples, such as *DNMT1*, *DNMT3A* and *TET3.* Bisulfite modification, cloning and sequencing were performed to study the KvDMR1 methylation patterns in four IUGR samples and in three control samples. Our sequencing results demonstrated a tendency to hypermethylation in IUGR samples, however, the difference between the two groups was non-significant. This tendency was corroborated by the overexpression of a gene that code the DNA methyltransferase in samples of fetuses diagnosed with IUGR. In addition to the absence of a significant difference between the two groups, it was observed that the association between gene expression, *CDKN1C* and *PHLDA2*, and the KvDMR1 methylation, although positive, was not significant.

The global hydroxymethylation of all samples was evaluated with a colorimetric method and the 5-hydroxymethylcytosine was present in placental samples. However, the results showed non-significant changes between the IUGR samples and control samples.

In conclusion, the results obtained during this thesis allow the identification of two potential biomarkers for IUGR. In addition to that finding, the study confirms the presence of hydroxymethylation in placenta and show a tendency for hypermethylation of KvDMR1 in IUGR samples. The absence of a significant association between gene expression and methylation can be justified by the action of another trans-acting mechanism. However, the tendency for hypermethylation of KvDMR1 and *PHLDA2* and *CDKN1C* increased expressions in IUGR samples show a possible association between those parameters and IUGR.Thus in further methylation studies, the sample size should be larger than that analyzed in this investigation.

Keywords

Epigenetic modification, Genomic imprinting, DNA methylation, DNA hydroxymethylation, Intrauterine growth restriction, qRT-PCR, Gene expression, Bisulfite genomic sequencing, 5-hmC DNA ELISA

Index

List of Tables

List of Figures

Abbreviations

BER	Base excision repair
Bp	Base pair
BWS	Beckwith-Wiedemann syndrome
BSA	Bovine Serum Albumin
CDK	Cyclin-dependent kinases
CDKN1C	Cyclin-dependent kinase Inhibitor 1C
cDNA	Complementary DNA
CNRQ	Calibrated normalized relative quantities
CpG	Cytosine-phosphate-Guanine dinucleotide
CPM	Confined Placental Mosaicism
CTCF	CCCTC-binding factor
CTG	Cardiotocography
DMR	Differentially methylated region
DNA	Deoxyribonucleic Acid
DNMT	DNA Methyltransferase
dNTP	Dinucleotide Tri-Phosphate
ELISA	Enzyme-Linked Immunosorbent Assay
ES	Embryonic Stem
FGR	Fetal Growth Restriction
Fw	Forward
HAT	Histone acetyltransferase
HDAA	Histone deacetylase
HKMT	Histone lysine methyltransferase
HRP	Horseradish Peroxidase
IC	Imprinting Centre
ICM	Inner cell mass
ICR	Imprinting Control Region
IGFBP	Insulin-Like Growth Factor-binding protein
IGF2	Insulin-Like Growth Factor 2
IMAGe	Intrauterine growth restriction, metaphyseal dysplasia, adrenal hypoplasia congenita, and genital anomalies
IUGR	Intrauterine growth restriction
KCNQ1	Potassium voltage-gated channel subfamily Q member 1
*KCNQ1*OT1	KCNQ1 overlapping transcript 1
lncRNA	long noncoding RNA
MEST	Mesoderm Specific Transcript Homolog
mRNA	messenger RNA
MS-MLPA	Methylation-Specific Multiplex Ligation-Dependent Probe Amplification
ncRNA	non-coding RNA
PAPP-A	Pregnancy-associated plasma protein A
PBS	Phosphate Buffered Saline
PBT	Phosphate-buffered saline with tween
PCR	Polymerase Chain Reaction

PEG10	Paternally Expressed 10
PHLDA2	Pleckstrin Homology-Like Domain, Family A, Member 2
PCNA	Proliferating cell nuclear antigen
PGC	Primordial germ cells
RQ	Relative quantities
qRT-PCR	Quantitative Real-Time PCR
Rv	Reverse
RT-PCR	Reverse Transcription PCR
RNA	Ribonucleic Acid
RPL0	ribosomal protein lateral stalk subunit P0
SGA	Small for Gestational Age
SAM	S-adenosylmethionine
SRS	Silver Russel Syndrome
TBP	TATA-Box Binding Protein
TDG	Thymine DNA glycosylase
TET	*T*en-Eleven Translocation
XCi	X chromosome inactivation
5-mC	5-methylcytosine
5-hmC	5-hydroxymethylcytosine

I. Introduction

1. Normal fetal development

Fetal growth, a complex and dynamic process, is linked to a linear growth and height in adult life. In addition to the physical influence, the intrauterine growth is also associated with young and adult work performance and with stillbirth (Uauy et al., 2013).

The normal growth is first determined by the length of pregnancy, the gestational age. A normal pregnancy lasts about 40.0 weeks (280 days) and a pregnancy is considered at term when the length is 37-42 weeks. A preterm pregnancy is when the length is less than 37.0 weeks and a post-term gestation is when the duration is more than 42.0 weeks (Gardosi, 2012). In addition to gestational age, the fetus size is a determinant of fetal development in which is assessed by ultrasound (Zhang et al., 2010).

The human fetal growth is usually based on anthropometric measurements at several weeks of gestation. In the first fifteen gestational weeks, the fetal weight rises about 5g per day and subsequently, the weight gain rate increases up to the thirty-fourth week. In the twentieth week, the weight acquisition is about 10g per day and it is substantially higher in the thirty-fourth week (30-35g per day). After this exponential increase, the gain weight rate is zero or negative. The weight gain variation indicates that the fetus needs are less in the first half of the pregnancy than in the second, at where the maximum weight is accomplished (Monk and Moore, 2004; Resnik and Creasy, 2014) (Fig.1).

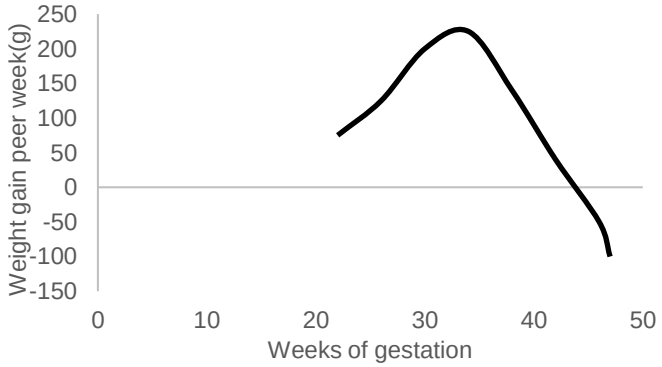


Figure 1 Growth rate curve for single births. Adapted from (Williams et al., 1982).

Normal fetal growth across the three trimesters is divided into three stages. The first occurs in the first half of pregnancy and it is distinguished from others stages by virtue of cellular hyperplasia. The increase of cell division leads to the development of the organ system. The establishment of tissue and organ pattern occurs in the first stage, followed by cellular adaptation and increase in body size. The increase in the number of cells also occurs in the second phase along with cellular hypertrophy, the increase in cell size. The last stage is characterized by hypertrophy growth in the last 6 to 8 weeks of pregnancy and occurs an organ systems maturation (Mullis and Tonella, 2008; Resnik and Creasy,

2014). The alteration of this dynamic fetal growth may cause the reduction of cell size and number, resulting in restriction of fetal growth (Baschat and Galan, 2016).

1.1 Fetal growth regulation

The fetal growth is affected by multiple factors related to the mother, the fetus, the placenta and the environment (Calkins and Devaskar, 2015). Deregulation of those factors can restrict the fetus normal growth and lead to higher risk of perinatal morbidity and mortality (Lyons, 2015). The placenta function is one of the factors that greatly modulate the fetal development, influencing the respiratory, hepatic and renal functions (Mayer and Joseph, 2013).

The genome of the fetus is the first factor that determines fetal growth, but the environment and the nutrition are also important in subsequent fetal development. In addition to genetic, environment and nutrition, the fetal growth throughout gestation is critically regulated by hormones and growth factors (Mullis and Tonella, 2008).

1.1.1 Endocrine regulation of fetal growth

Hormones can stimulate or inhibit fetus growth in utero. Insulin, insulin-like growth factors I and II, and glucocorticoids are essential hormones for fetal growth and development that respond to metabolic, endocrine and neural stimuli (Fowden and Forhead, 2009). These hormones regulate the availability of nutrients for fetal growth and also regulate tissue growth and differentiation, acting as environmental signals (Fowden et al., 2015). The interaction between the growth inhibitory hormones and the growth stimulatory hormones allows the control of fetal and placental growth. The regulation of growth by hormonal factors involve the interaction between the mother, the fetus and the placenta. Insulin is a growth hormone, in which its deficit causes growth restriction due to decreased absorption and utilization of nutrients by the fetus (Murphy et al., 2006). The mitogenic effect on cellular development makes insulin a cell number controller hormone (Sharma et al., 2016b).

The Insulin-like growth factor (IGF) system is associated with regulation of fetal and placental growth, and with IGFs stimulate growth in utero. The IGF ligands (IGF-I and IGF-II) bind to type 1 receptors (IGF-1R), resulting mainly in mitogenic and anti-apoptotic effects. This type 1 ligand is regulated by glucose. The IGF type 2 receptor (IGF-2R) mediate the endocytosis of IGF-II and its degradation in lysosomes, controlling extracellular IGF-II. In addition to IGF ligands and receptors, the IGF system includes binding proteins (IGFBPs), that bind IGF ligands and modulate its effects (Gicquel and Le Bouc, 2006). During gestation, the endocrine regulation of fetal growth is made mainly by the primary hormones, insulin and IGF-I, and also by IGF-II. IGF-I is important during late pregnancy and the increase in serum levels is observed over gestational age. Both IGF-I and insulin seem to be related to the delivery of nutrients. The IGF-II levels increase during mid- to late gestation and its serum levels are much higher than serum IGF-I levels, supporting the embryonic growth (Mullis and Tonella, 2008). The local action of IGF is increased when Pregnancy-associated plasma protein-A (PAPP-A) is present. The stimulatory role occurs due to the PAPP-A function in cleave IGFBP-4, an inhibitor of IGF action (Sharma et al., 2016b) (Fig.2).

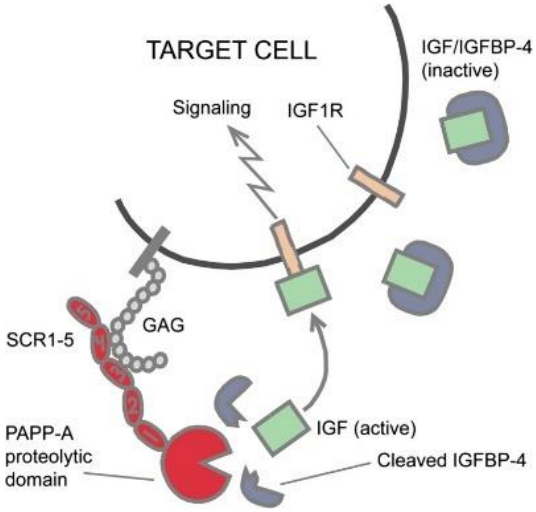


Figure 2 Function of PAPP-A and IGFBP-4 in the IGF system. PAPP-A binds to proteoglycans on the cell surface, causing the cleavage of IGFBP-4. Subsequently, IGF receptor binds to IGF, leading to receptor signalling. SCR-Small consensus repeat; GAG-Glycosaminoglycan; PAPP-A- Pregnancy-associated plasma protein-A; IGF-Insulin growth factor; IGFBP-Insulin growth factor binding protein (Oxvig, 2015).

In addition to growth stimulatory hormones, the fetal growth is also regulated by inhibitory hormones. The glucocorticoids are the main hormones that directly inhibit the fetal development. Those inhibitory hormones are responsible for maturation of key fetal tissues for neonatal survival, such as liver, lungs, gut, skeletal muscle and adipose tissue. They activate physiological systems, that have a diminutive function in utero, and their action occurs particularly close to term, preparing the fetus for extrauterine life (Fowden and Forhead, 2015). The early-life administration of glucocorticoids leads to a

reduction of fetal tissues, resulting in fetal growth restriction. The effect of fetal growth reduction is mediated, partially, by variations in placenta, affecting placental nutrient transfer and the production and metabolism of hormones (Fowden and Forhead, 2009). In addition to production and transport of growth stimulatory hormones, the placenta also functions as a barrier to prevent high concentrations of glucocorticoids from the mother (Murphy et al., 2006).

1.1.2 Placental regulation of fetal growth

Fetal growth is severely modulated by placental function. The placenta is a fetal-maternal endocrine organ that enables the exchange of nutrients and waste products between the mother and the fetus (Frost and Moore, 2010). This organ is responsible for the allocation of oxygen and nutrients to the fetus, and at the same time, for the transfer of residual products and carbon dioxide to the mother (Gude et al., 2004) (Fig.3).

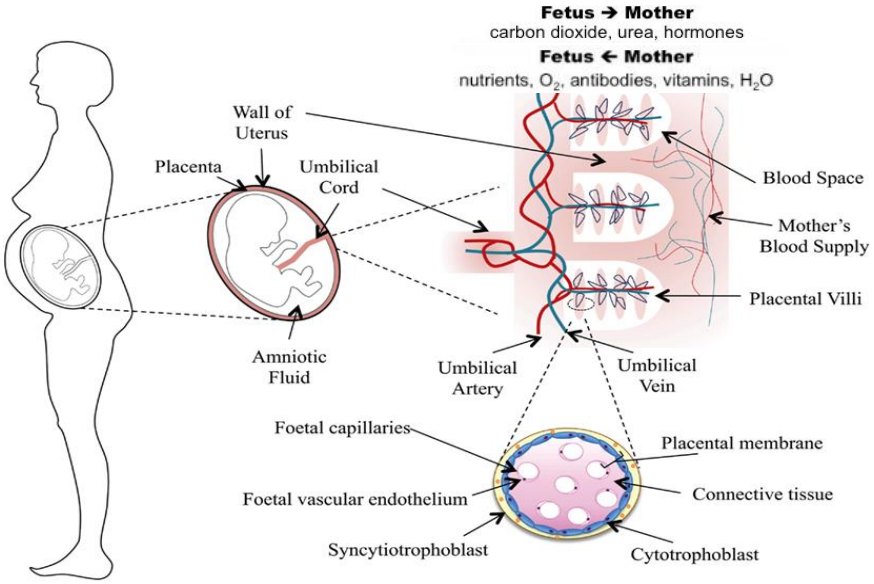


Figure 3 Schematic illustration of mother-fetus interactions through the placenta and placenta structure (Stolp et al., 2012).

Furthermore, the placenta has the important functions of protecting the fetus from mother's immune system and secreting hormones and growth factors (Maccani and Marsit, 2009). Thus, for an excellent placental function and normal fetal growth, it is necessary a correct placental development, which begins with placentation.

The placenta is constituted by both fetal tissue and maternal tissue, the fetal chorion (resulting from blastocyst) and maternal decidua (resulting from changes that occur in endometrium) (Gude et al., 2004; Monk, 2015). The interaction between maternal and embryonic cells allows interstitial implantation and formation of the placenta (Gude et al., 2004). Placentation initiates with the fusion of trophoblast cells, resulting in a two-layered

structure of syncytiotrophoblast and cellular cytotrophoblast. Subsequent to this event, protrusions of syncytiotrophoblast attach and penetrate to the decidualized endometrium, following the formation of vascular connections that enable efficient maternal–foetus exchange (Frost and Moore, 2010)(Fig.4). Studies have proposed that the processes of implantation and early placentation are important to determine the fetal growth disorder, once the invasion of trophoblast occurs during the five months of gestation and it is a continuous process (Smith and Lees, 2012).

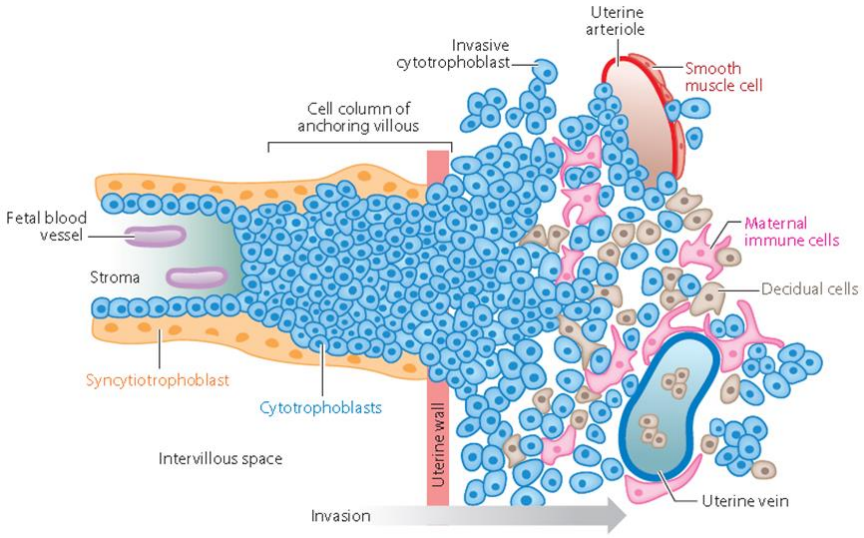


Figure 4 Formation of placenta after trophoblast implantation (Maltepe and Fisher, 2015).

Generally, the growth of placenta precedes fetal growth and the abnormal placental growth is related to restriction of fetal growth (Regnault et al., 2001). The placental control of fetal growth is variable and the growth of fetus can be not proportional to placental development.

Factors related to the placenta, such as abnormal placental transfer of oxygen and nutrients, anomalous cord insertion, chorioangiomas(benign tumours of the placenta), the presence of a single umbilical artery, alterations on fetoplacental blood flow, concentration gradients of nutrients and placental metabolism, disturb the normal development of placenta and fetus (Beard and Nathanielsz, 2013; Gaccioli and Lager, 2016). In addition to those factors, the thickness and exchange area of the placenta may also have effects on fetal growth, influencing the transfer of molecules across the placenta (Gaccioli and Lager, 2016).

Placental and fetal size are correlated with each other, although interrelationships between the fetus and the placenta are essential for fetal growth and development. The transfer of substances between the maternal and fetal circulations, separated by two cell layers, can be influenced by several factors (Regnault et al., 2001). One of the crucial

aspects influencing the nutrients exchange is the placenta transport capacity (Gaccioli and Lager, 2016).

1.1.3 Genetic regulation of fetal growth

Genetic is the primary factor that controls fetal growth rate by an intrinsic fetal growth potential. The determination of normal fetal growth and size at birth by genetic factors predominates during the first half of pregnancy. In 2006, it was determined that the genetic inheritance contributes in 30% to 70% to birth weight (Dunger et al., 2006).
Studies in twins verified that final weights were highly associated in monozygotic twins, but not the same was observed in dizygotic twins, indicating the role of genes in regulating fetal growth (Kurjak and Chervenak, 2006).
Genetic regulation in fetuses with a normal growth and size at birth is mediated by multiple gene loci. Nevertheless, single gene loci can also influence fetal birth weight. Genetic variations in genes coding for IGF-I, IGF-II, insulin and their respective receptor are related with fetal development (Zhang et al., 2010).
In addition to single loci variations, the fetal growth is also influenced by chromosomal abnormalities. The reduction of cell multiplication rate, resulting from chromosomal abnormalities, such as trisomy 21, trisomy 18, trisomy 13, 4p syndrome and monosomy X, are related to reduced birth weight (Beard and Nathanielsz, 2013).
Fetal growth is predominantly influenced not only by the fetal but also by the maternal genotype. During fetal development, the paternal and maternal genotypes contribute to fetal autosomal and sex genes by spermatozoa or oogonia. The maternal regulation of growth is made not only by the contribution of fetal autosomal and sex genes but also by the genotype effect on the fetal environment (Regnault et al., 2001).

2. Fetal growth disorder

Disturbance in factors related to the fetal regulation can result in a fetal growth disorder. The conditions in which occurs the failure of a fetus to reach the growth potential are fetal growth disorders. The growth disorders of the fetus are determined considering the expected dimensions of fetus relative to the age of gestation. The measurements can be accessed directly at birth or using ultrasound if the interest is the measurement during the fetal life (Smith and Lees, 2012).
The intrauterine growth restriction (IUGR), a fetal growth disorder, is the second leading cause of perinatal mortality, following the prematurity (Baschat and Galan, 2016).

2.1. Intrauterine growth restriction

Intrauterine growth restriction (IUGR) is a condition in which the fetus is not able to achieve its normal growth potential (Baschat and Galan, 2016).

The growth restriction of fetus affects approximately 5-15% of all pregnancies in the United States and Europe. In developing countries, such as South Central Asia, Africa and Latin America, this percentage increase but is very variable (Gaccioli and Lager, 2016). The incidence of IUGR in underdeveloped can be six times higher than in developed countries, differing among countries, populations and races. The Asian population is the one that presents more cases of IUGR, approximately 75% of all IUGR infants (Sharma et al., 2016b).

In literature, several definitions of IUGR are found and some of those are associated to the fetal weight estimation for gestational age (<25 %, <15 %, <10 %, <5 %, <3 %, <2.5 %, and <1 %). Others definitions include more parameters for assessing the disorder, such as the abdominal circumference <10% for gestational age, or the deviation or reduction in an expected fetal growth pattern (Suhag and Berghella, 2013; Sharma et al., 2016b).

IUGR is associated with relevant clinical characteristics, like malnutrition and in-utero growth retardation. This condition is classified as symmetrical or asymmetrical. IUGR is symmetrical when weight, length and head circumference are proportionally reduced. Approximately 70%-80% of IUGR cases are asymmetrical and in that case, IUGR is characterized by a higher reduction in weight than the length and head circumference (Calkins and Devaskar, 2015). The symmetrical restriction growth typically results from a factor (predominantly aneuploidy, malformations, or less commonly, fetal infection) that influences cell division in early pregnancy, leading to the decrease in size and number of cells (Resnik and Creasy, 2014; Baschat and Galan, 2016). On the other hand, the asymmetrical restriction growth results from two processes. First, the limited nutrient supply leads to depletion of glycogen stores and result in a liver volume reduction. Second, the occurrence of an increase of blood and nutrient supply to structures in the upper part of the body, culminating in “head sparing” (Baschat and Galan, 2016). In addition to symmetrical and asymmetrical IUGR, a third type emerged to classify newborns that have clinical features of both types of IUGR, called mixed IUGR. In this type of IUGR a smaller number of cells and small size cells are observed (Sharma et al., 2016b).

2.1.1. Diagnosis of IUGR

IUGR is related to an increase in the risk of perinatal mortality and morbidity, therefore, the cause of intrauterine growth retardation should be determined early in pregnancy (Peleg et al., 1998). The determination of IUGR causes, before delivery, allows the appropriate counselling, delineate fetal anatomy, screen growth velocity and obtain neonatal consultation (Resnik and Creasy, 2014).

Physical examination is one of the first steps in IUGR diagnosis and it uses techniques that examine fetal growth, with indirect or direct measures. Maternal weight gain, fundal height and correct fetal gestational age are considered indirect measures. The direct measurement could be obtained with obstetrical ultrasound (Lyons, 2015). Ultrasonography is the diagnosis technique most preferred and accepted for the diagnosis of fetal growth restriction. This procedure estimate fetal weight, determine fetal growth velocity and measure fetal dimensions (Resnik and Creasy, 2014).

Physical examination, by itself, is not sensitive and accurate for IUGR diagnosis, since this pathology can be the consequence of many subjacent factors. Considering that, a differential diagnosis should be made, in which it is screened specific aspects of the patient's risk factors that may contribute to growth restriction (Albu et al., 2014; Resnik and Creasy, 2014). Screening tools, like patient's history, physical evaluation and laboratory tests, are useful for diagnosis of IUGR (Lausman et al., 2012). Furthermore, others evaluation criteria should be taken into account, such as the measures of the mother's body, maternal nutritional assessment, gestational dating, fundal height with fetal palpation, cardiotocography (CTG), ultrasound with Doppler (uterine and umbilical artery), and fetal weight measurement using biometric measures (Sharma et al., 2016b).

2.1.2. IUGR consequences

One of the sequelae of IUGR is stillbirth and usually results from placental insufficiency (Saleem et al., 2011). In addition, fetuses with restricted growth have both short-term and long-term complications after birth, and those are related to the cause of the growth defect (Calkins and Devaskar, 2015; Sharma et al., 2016b). The short-term complications include neonatal asphyxia, meconium aspiration, hypoglycemia, hyperglycemia, hypothermia, pulmonary persistent hypertension, polycythemia, hypocalcemia, and other metabolic abnormalities (Resnik and Creasy, 2014; Calkins and Devaskar, 2015; Sharma et al., 2016b). The long-term consequences comprise poor growth, neurodevelopment outcome and more increased susceptibility to degenerative diseases, such as diabetes, cardiovascular problems and hypertension (Saleem et al., 2011; Demicheva and Crispi, 2014). The neurodevelopment impairments associated

with this condition include cerebral palsy and domains of cognition, attention, mood and social skills (Calkins and Devaskar, 2015).

2.1.3. IUGR causes

Intrauterine growth restriction is caused by highly heterogeneous factors that affect normal intrauterine growth. The causative factors can be divided into, placental, environmental, maternal and fetal factors.

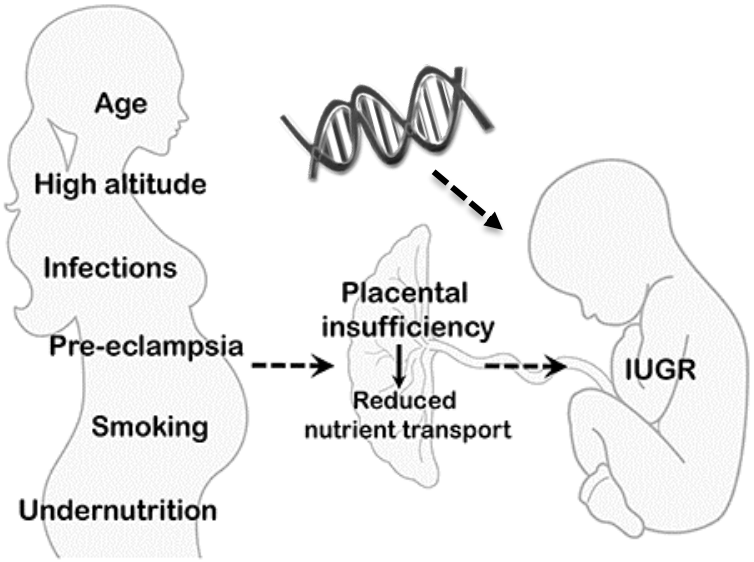


Figure 5 Maternal, environmental, placental and genetic conditions associated with IUGR. Adapted from (Gaccioli and Lager, 2016).

a. Maternal and environmental factors

The leading cause of IUGR is a maternal vascular disease in association with preeclampsia and impaired uteroplacental perfusion, that accounts between 20% to 30% of IUGR (Bamberg and Kalache, 2004; Resnik and Creasy, 2014). Medical complications in the mother that affect uteroplacental blood flow, such as hypertension, renal insufficiency, anaemia and diabetes, may also result in IUGR. Other mothers diseases leading to growth restriction include pulmonary disease, systemic lupus erythematosus, thrombophilia and antiphospholipid syndrome (Krishna and Bhalerao, 2011; Calkins and Devaskar, 2015).

Maternal exposure factors that may contribute to growth restriction disorder, comprise the age of the mother, substance abuse (smoking, alcohol and drugs), malnutrition, maternal infection and the mother's country of origin (Monk and Moore, 2004; Sharma et al., 2016b). All the factors that contribute to an irregularity in placental blood flow can lead to IUGR. An example of that is mothers that live in high altitudes regions, resulting in a reduced blood volume and reduced oxygen carrying capacity (Sharma et al., 2016a).

b. Fetal factors

Genetic variations, structural malformations and infections are fetal causes that result in IUGR (Hendrix and Berghella, 2008).

Genetic variations can influence the growth of fetus both directly and through effects on the placenta. Over recent years, the genetic causes of IUGR are increasing and better studied with improved knowledge of molecular techniques. The genes involved in this disorder, that encode for proteins or hormones, may be of maternal, fetal and placental origins (Sharma et al., 2017). Chromosomal abnormalities, including trisomy 13 (Patau syndrome), 18 (Edwards syndrome) and 21 (Down syndrome), are strongly associated with IUGR (Monk and Moore, 2004). In patients with Edwards syndrome is observed growth restriction more severe than in the other two trisomies. Although the abnormalities in sex chromosomes are usually lethal, they are also related to growth retardation in survivors. The incidence of chromosomal anomalies in IUGR infants varies from 7% to 20%. The rise of percentage to 20% occurs when the disorder is diagnosed in the first half of pregnancy (Bamberg and Kalache, 2004). Some fetal growth retardation cases are caused by confined placental mosaicism (CPM), a chromosomic mosaicism in which the placental karyotype have mosaicism for an abnormality and the karyotype of the fetus is normal (Wilkins-Haug et al., 2006). CPM is present in trisomy 16, which is usually lethal in the cases that mosaicism is not observed, resulting in IUGR (Hendrix and Berghella, 2008).

In addition to chromosomal abnormalities, single-gene defects and aberrant expression of imprinted genes are also genetic variations associated with IUGR. Genes polymorphisms have been linked to the restriction of fetal growth, which is the case of *IGF1* and *IGF2* (Sankaran and Kyle, 2009). Studies related the aberrant expression of *IGF2* gene and other imprinted genes to this disorder (Moore et al., 2015). The aberrant expression of imprinted genes, which promotes or restricts fetal growth, underlines the important role of imprinting in the regulation of fetal growth (Smith and Lees, 2012).

Congenital intrauterine infections are other fetal origins of IUGR, representing between 5% to 10% of IUGR cases. Studies associated infection of rubella, cytomegalovirus, varicella and herpes zoster with early-onset intrauterine growth restriction and the consequences are severe (Resnik and Creasy, 2014). The first two inhibit cell division and promote cell death, resulting in a reduction in cell number and subsequently in IUGR (Grimberg and Lifshitz, 2007).

c. Placental factors

Placental insufficiency and multiple gestations are placental factors that can lead to IUGR, affecting nutrient and oxygen delivery to the placenta and transference of those

components across this organ (Calkins and Devaskar, 2015). Furthermore, the conditions can influence on nutrient uptake by the fetus and on the regulation of growth processes (Baschat and Galan, 2016). The majority of IUGR cases are, directly or indirectly, due to placental insufficiency. This primary cause occurs due to alterations in uteroplacental and fetal-placental circulations, which commonly comes from the inadequate trophoblastic invasion of the spiral arteries (Krishna and Bhalerao, 2011). Features like, abnormalities of the maternal spiral arterioles, dysregulated villous vasculogenesis, and abundant fibrin deposition, are directly observed in placental dysfunction (Scifres and Nelson, 2009).

Multiple gestation is linked with a decrease in fetal and placental weight and is observed a discordant growth in twins. In monochorionic twins, twins that share the same placenta, the discordant growth is higher than in dichorionic twins (Figueras and Gardosi, 2011; Resnik and Creasy, 2014).

3. Epigenetic modifications

The function and phenotype of all cells in the human body are different, although they have the same genome. This phenomenon is possible due to the epigenetics, the study of alterations in phenotype or gene expression without alteration in the DNA sequence (Nelissen et al., 2011).

Placental development and fetal growth can be regulated by epigenetic modifications, also named epimutations (Monk, 2015). Epigenetic marks are reversible modifications, such as DNA methylation and chromatin modifications, that influence gene transcription by shaping genome architecture and accessibility to transcription factors (Bianco-Miotto et al., 2016). The epimutations do not belong to the genome and they are specific to the type of cell and stage of development. The epigenetic marks are deposited early in development, but during life, the marks may adjust in response to stimuli. The environment has a large influence in this type of modifications and can cause abnormal placental development and function (Januar et al., 2015).

In the mammalian development, the epigenetic reprogramming occurs two times, during the generation of the female and male germ cell, and in early post-fertilization. In the first stage is observed a demethylation event, where occurs the removal and the establishment of specific epigenetic marks. After fertilization and prior implantation, the zygote suffers several cellular divisions and reach the blastocyst stage. During that time, the DNA methylation decreases and the genome is almost completely hypomethylated in the blastocyst stage, in which the imprinted epigenetic marks continue intact (Januar et al., 2015). In addition to DNA demethylation, during preimplantation development occurs the reorganization of histone modifications. The epigenetic reprogramming in the blastocyst stage is distinct between the two lineages, that result from totipotent blastocyst cells differentiation. The differentiation in pluripotent inner cell mass (ICM) and trophectoderm (TE), arising the fetus and extraembryonic tissue, respectively, is possible due to distinct epigenetic alterations. The variation in epigenetic result in changes in gene expression between the two lineages. In extraembryonic tissue, such as the placenta, is observed hypomethylation, when compared with somatic cells. Furthermore, evidences suggests that in the ICM there is a greater abundance of histone methylation (Morgan et al., 2005; Januar et al., 2015). Therefore, the global modifications of epigenetic marks, that occurred in development, contribute to limit the cellular potential and the establishment of cell lineages (Guibert and Weber, 2013).

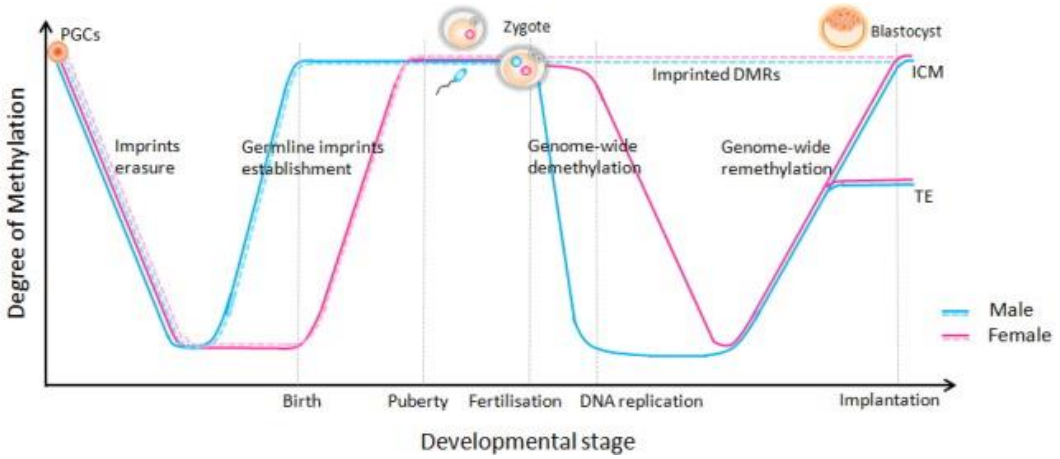


Figure 6 DNA methylation during gametogenesis and early embryonic development. DMR- differentially methylated region; ICM- Inner cell mass; TE- trophectoderm (Ishida and Moore, 2013).

3.1. DNA methylation

The most widely characterized epimutation is the DNA methylation. This epigenetic modification consists in the incorporation of a methyl group (-CH_3) to 5'-carbon of the cytosine bases, resulting in 5-methylcytosine (5-mC) (Yuen et al., 2009).

This process is mainly catalyzed by differents DNA methyltransferases (DNMT) enzymes, the DNMT1, the DNMT3A, the DNMT3B and the DNMT3L, and requires a methyl donor, the S-adenosylmethionine (Fig.7). The DNMTs are central for the methylation maintenance and embryonic development but the enzymes have different rules. The DNA methylation maintenance and repair is predominantly achieved by DNMT1, restoring the methylation pattern on hemimethylated substrates during DNA replication. The inhibition of DNMT1 may result in passive demethylation. Others DNMTs (DNMT3A,DNMT3B and DNMT3L) have an essential role during early development, the establishment of *de novo* DNA methylation, acting on hemimethylated and unmethylated DNA (Kinney and Pradhan, 2011; Bianco-Miotto et al., 2016).

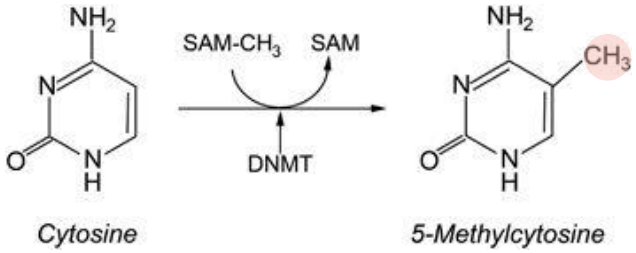


Figure 7 Conversion of cytosine to 5-methylcytosine by DNA methyltransferase (DNMT). S-adenosylmethionine (SAM) is the methyl donor (Gibney and Nolan, 2010)**.**

The methylation of DNA occurs frequently in CpG dinucleotides, which is a cytosine directly followed by a guanine. In normal cells, the percentage of methylated CpGs is nearly 80%. Regions of DNA sequence that have a rich content of CpG dinucleotides are termed CpG islands. These regions are usually associated with the promoter region of the gene (nearly 60% of the genes) and comprise 1-2% of the genome. CpGs islands in promoter regions are important for transcriptional regulation (Koukoura et al., 2012).
DNA methylation is associated with silencing gene expression and maintenance of genome stability. Normally, although with exceptions, gene expression silencing or gene expression reduction occurs when the cytosines at CpG dinucleotides are methylated in the promoter regions of genes. On the other hand, if the cytosines are not methylated the opposite is observed, resulting in gene transcription and expression. The repression of gene transcription occurs due to the non-recognition of promoters by transcription factors and RNA polymerase, resulting from the binding of several elements (for example methyl CpG binding protein-MeCP) to methylated DNA (Maccani and Marsit, 2009). The methylation of CpG dinucleotides in gene promoter regions together with histone protein modifications leads to chromatin compaction, reinforcing gene silencing. In normal conditions the gene promoters have most of the CpG islands unmethylated, allowing active gene transcription (Bird, 2002; Koukoura et al., 2012).

The methylation of DNA may be biallelic, occurring in both alleles, or monoallelic. The monoallelic methylation is present in two epigenetic phenomena, the genomic imprinting and the X inactivation. The silencing of one allele is only observed in both genomic imprinting and the X inactivation, however, the first is parental origin specific (Bird, 2002). DNA methylation is crucial for embryo and placenta development and the presence of 5-mC on promoter regions of some gene is associated with a reduction in the expression of the genes (Green et al., 2016). The imprinted and non-imprinted gene expression is controlled by the methylation of those genes promoters, influencing fetal growth. Studies in rats demonstrated that the administration of a DNA methyltransferase inhibitor to pregnant rats resulted in a significant reduction in placentas and in the labyrinthine part of the placenta (Koukoura et al., 2012).

The placenta is comprised of different cell types, predominantly the villous syncytiotrophoblast. This type of trophectoderm-derived cell exhibits a DNA with low methylation level, which contributes for the hypomethylation observed in the placental genome (Schroeder and LaSalle, 2013).

In addition to the variation of methylation levels in the diverse types of placental cells, others parameters, such as fetal gender, gestational age and pregnancy complications, may contribute to the change in placental methylation levels (Bianco-Miotto et al., 2016).

3.2. DNA hydroxymethylation

Besides 5-methylcytosine, other cytosine modification present at differentially methylated regions (DMRs) have been described in the placental genome, the 5-hydroxymethylcytosine (5-hmC). In the DNA demethylation process, the 5-methylcytosine oxidation gives rise to 5-hydroxymethylcytosine and it is controlled by the ten-eleven translocation (TET) family of proteins. The TET proteins are 2-oxoglutarate and Fe(II)-dependent dioxygenases that use α-ketoglutarate as a cosubstrate for the conversion (Guibert and Weber, 2013). TET1 was the first protein associated with hydroxymethylation, possessing the DNA-binding domain CXXC (that recognize CpG site). The TET2, in contrast with TET1 and TET3, lacks this domain, and the recruitment to target genes is helped by the TET2 ancestral CXXC domain (IDAX) (Ko et al., 2013). After the conversion into 5-hmC, it may occur a passive demethylation upon DNA replication or an active demethylation (Chen and Riggs, 2011). In addition to conversion into 5-hmC, the TETs proteins are also responsible for 5-mC oxidation into 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC), with the intermediate conversion in 5-hmC. These two modified bases, present in DNA from Embryonic Stem (ES) cells and early embryos, can be removed from DNA by the action of thymine DNA glycosylase

(TDG). Subsequently to TDG action occurs base excision repair (BER), resulting in a cytosine by active demethylation (Guibert and Weber, 2013) (Fig.8).

Recently, studies suggest the correlation between gene expression and the epigenetic modification 5-hydroxymethylcytosine in actively transcribed genes (Green et al., 2016). In addition to the association with active genes, 5-hmC has been related with demethylation pathways and may serve as an epigenetic mark (Chapman et al., 2015). This epigenetic modification may have an additional function of modulating the binding of chromatin effectors, influencing gene expression. Although the 5hmC role is not evident, researchers verified the presence of that epigenetic modification in some placental imprinted loci and the association with size at birth (Piyasena et al., 2015). In some studies, the 5hmC presence was not considered and it can be misrepresented as 5-mC. For that reason, it is important to better understand the 5-hmC distribution, distinguish between 5-hmC and 5-mC, and investigate a potential role for this modification.

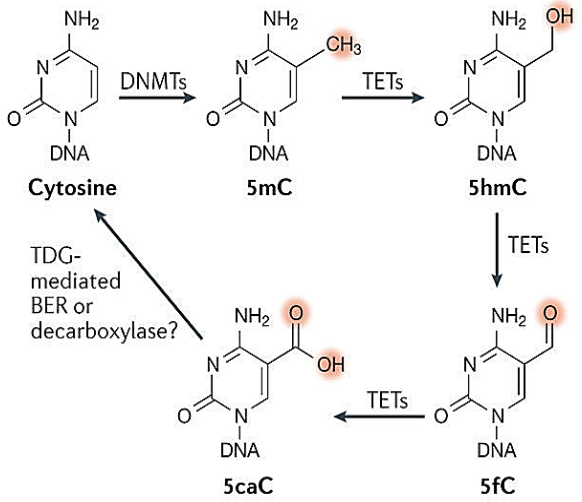


Figure 8 DNA Methylation, hydroxymethylation, and oxidative demethylation. DNMT- DNA methyltransferase; TET-Ten eleven translocation; 5mC-5 methylcytosine; 5fC-formylcytosine; 5-caC-5-carboxylcytosine; TDG- thymine DNA glycosylase; BER- Base excision repair (Zhu et al., 2016).

3.3. Histone modification

Histones are a family of small proteins that bind to DNA very tightly, forming a DNA-protein complex called chromatin. Gene expression is regulated by modifications of the chromatin environment (Maccani and Marsit, 2009).

In 1964, Vincent Allfrey observed that histones are post-translationally modified (Allfrey et al., 1964). The modification of histones may be from several types, including phosphorylation, ubiquitination, acetylation and methylation. The covalent modifications of these positively charged proteins regulate chromatin structure and that may influence transcription (Maccani and Marsit, 2009). The chromatin structure is affected due to the

alteration of the highly basic histone amino (N)-terminal tails, which normally extends from the nucleosomes and interact with the adjacent nucleosomes. The histones modifications recruit proteins and complexes with specific enzymatic activities, affecting internucleosomal interactions, and thus, the chromatin structure (Bannister and Kouzarides, 2011). In addition to regulation of gene expression, histones play a crucial role in DNA repair, recombination and replication, consequently histones modifications can also affect those processes (Lennartsson and Ekwall, 2009).

The first histone modification reported was histone acetylation, the addition of acetyl group to lysines (Allfrey et al., 1964). Histone acetyltransferases (HATs) and histone deacetylases (HDACs) are the families of enzymes that regulate the acetylation of lysines and they have opposite effect in lysines residues. The first family neutralize the lysine's positive charge, catalyzing the allocation of an acetyl group to the ε-amino group of lysine side chains. This acetyl transfer affect interactions between histones and DNA (Bannister and Kouzarides, 2011). The HDACs family remove the acetyl group from the ε-amino group of lysine side chains, restoring the positive charge of the lysine. In genome, the acetylation is present at low levels, probably due to the equilibrium between HAT and HDAC activities (Vaissière et al., 2008).

Histones phosphorylation is the addition of the phosphate group to the serines, theorines and tyrosines, which are commonly present in the N-terminal tails of histones. The regulation of phosphorylation of histones is made by enzymes families that add and remove phosphate group of the hydroxyl group of the amino acid target, the kinases and phosphatases, respectively (Bannister and Kouzarides, 2011).

Another modification of histones is histone methylation that occurs in the side chains of lysines, arginines and more rarely histidines. This process is catalyzed by histone lysine methyltransferase (HKMT) and arginine methyltransferase enzymes that added a methyl group to lysines and arginines, respectively. Histone methylation is a reversible process and the methyl group removal is achieved by the action of histone demethylases (Greer and Shi, 2012).

Lastly, the ubiquitylation of histones is the ubiquitin attachment to histones lysines. The ubiquitylation modification differs from the others previously mentioned modifications because it is a large covalent alteration, being thus added a 76-amino acid polypeptide to histone lysines (Bannister and Kouzarides, 2011). The modification by ubiquitination of lysine is a reversible process, in which the modification is removed via the action of deubiquitylase. The binding of the modification is achieved via the action of ubiquitylases (Nelissen et al., 2011).

3.4. Non-coding RNA (ncRNA)

Variation in gene expression may occur due to the function and interactions of RNA molecules. In mammalian genome, most of the transcripts are non-coding RNAs (ncRNAs), RNA molecules that are not translated into proteins (Nelissen et al., 2011). These transcripts were first considered as “junk” or artefacts but currently, it is known that they can be functional.

ncRNAs can be classified based on their size, in short ncRNA and long ncRNA. The first term is applied when the ncRNA length is less than 200 nucleotides, while larger transcripts are named as long ncRNA (lncRNA) (Peschansky and Wahlestedt, 2014). RNAs like short interfering (si) RNAs, micro (mi) RNAs, piwi-interacting RNAs and short nucleolar(sno) RNAs, are examples of short ncRNAs (Nelissen et al., 2011). On the other hand, long ncRNAs can be grouped in five non-exclusive categories, the natural antisense transcript (NAT), long intergenic noncoding RNA (lincRNA), stand-alone lncRNAs, divergent transcripts, promoter-associated transcripts, and enhancer RNAs, and pseudogenes (Kung et al., 2013).

According to their function, the ncRNA can have Cis-acting functions, regulating the expression of one or more genes on the same chromosome, or Trans-acting function, regulating the expression of one or more genes on diverse chromosomes or regulating mature RNAs in the cytoplasm. Frequently, the ncRNA that acts in *cis* are long non-coding RNA and those who act in *trans* are short non-coding RNAs.

The X chromosome inactivation (XCI) in females is associated with two lncRNA, the Xist (inactive X-specific transcript) and Tsix (X-specific transcript) (Nelissen et al., 2011). In the genomic imprinting is also involved lncRNAs, such as the antisense lncRNA Kcnq1ot1 and the intergenic lncRNA H19. This RNAs repress flanking genes promoters in cis, resulting in the regulation of expression of adjacent genes (Kanduri, 2016).

4. Genomic imprinting

In the 80s, nuclear transplantation experiments showed that when two female pronuclei or two male pronuclei originated diploid mouse embryos these were not viable, discovering the imprinted genes (McGrath and Solter, 1984; Surani et al., 1984). Those studies were crucial to understanding the essential role of both maternal and paternal genomes for the embryonic development and the non-equivalency between those genomes (Ishida and Moore, 2013).

Genomic imprinting is an epigenetic process that causes silencing of one allele and results in monoallelic expression, according to the parental origin of the allele. This

process does not change the DNA sequence and, although it can be reversible, it is a heritable modification (Piedrahita, 2011; Ishida and Moore, 2013).

The expression of imprinted genes is regulated by epigenetic modifications that are acquired during gametogenesis. These epigenetic modifications include cytosine methylation, histone tail modifications and more recently was discovered hydroxymethylcytosine (Lim and Ferguson-Smith, 2010; Guibert and Weber, 2013).

The imprinted genes can be located in clusters and frequently contain an imprinting control region (ICR), regulators elements of imprinting located in gene promoters or in intergenic regions (Maccani and Marsit, 2009; Piedrahita, 2011). The differential marking, observed in genomic imprinting, involves DNA methylation, DNA hydroxymethylation and histone modification at ICRs. These regions have differentially methylated regions (DMRs), CpG rich regions where the differential DNA methylation occurs. In germline, the ICRs are DMRs and differential methylation occurs during gametogenesis (Lim and Ferguson-Smith, 2010).

The epigenetic information is erased in the primordial germ cells (PGCs), which are the gamete precursors, occurring the passive demethylation of the genome. In male germ cells, *de novo* DNA methylation began subsequent to mitotic divisions of spermogonia. On the other hand, the *de novo* methylation in oocytes initiates during meiosis. During this time, the sex specific methylation is established in imprinted genes. After fertilization, the paternal and maternal pronucleus undergoes a genome-wide demethylation. The demethylation is active in the paternal pronucleus, involving TET family enzymes and 5-hydroxymethylcytosine. The passive demethylation mechanism is observed in the maternal pronucleus, depending on the DNA replications. Although the parental genomes undergo demethylation, imprinted methylated genes remain methylated, probably resulting from a mechanism in which maternal protein Developmental Pluripotency Associated 3 (DPPA3) protect those regions. Subsequently, around the time of implantation, both paternal and maternal genomes are remethylated and occurs the maintenance of methylation marks. During the imprinting cycle, all mechanism of *de novo* methylation and maintenance is achieved by the action of DNA methyltransferases (DNMTs) enzymes family (Ishida and Moore, 2013; Hitchins, 2015).

Genomic imprinting is observed in placental mammals and in flowering plants that possess an endosperm with placenta-like function (Ishida and Moore, 2013). Therefore, it is known that this phenomenon plays a key role in fetal development and placentation (Bressan et al., 2009). Several hypotheses have been developed to explain the emergence of genomic imprinting in placental mammals. The theory most accepted is “parental conflict theory”, which postulates that paternally expressed genes support nutrients extraction from the mother, enhancing fetal growth. In contrast, maternally

expressed genes restrict nutrient provision and assure not only her survival but also the equal supply of resources for her offspring (Bressan et al., 2009; Lambertini et al., 2012; Moore et al., 2015). Imprinted genes balance the maternal and paternal conflicting interests at all stages of development, including fetal life (Smith and Lees, 2012).

The intrauterine environment can be affected by injuries, leading to different levels of placental genes expression. A large number of placental genes are epigenetically regulated and the alterations of that marks may cause placental dysfunction, culminating in detrimental pregnancy complications such as intrauterine growth restriction (Lee and Ding, 2012). This condition affects, for example, the placentation of first-trimester placental trophoblast cells and leads to uteroplacental insufficiency (Moore et al., 2015). Therefore, a proper placental development and the capacity to compensate the injury are essential for a normal fetal growth (Scifres and Nelson, 2009; Sharma et al., 2016b).

4.1. Imprinted genes in IUGR placentas

In the human genome, approximately 150 imprinted genes are related to fetal and placental growth (López-Abad et al., 2016).These genes are essential for fetal and placental development and an aberrant gene expression is related to various disorders, including multifactorial humans diseases (Monk, 2015). An aberrantly functioning placenta can lead to an abnormal imprinted genes expression in placenta, resulting in IUGR (Frost and Moore, 2010). Thus, to provide more precise therapeutic options it is utmost importance to understand the effect of imprinted genes in fetal growth and development.

In the last decades, some studies analyzed gene expression in IUGR cases or the relation between gene expression and decreased birth weight, and they demonstrated not only the upregulation of some imprinted genes, such as *CDKN1C* (Cordeiro et al., 2014; Piyasena et al., 2015; López-Abad et al., 2016), *PHLDA2* (McMinn et al., 2006; Diplas et al., 2009; Janssen et al., 2016), *KCNQ1* (Cordeiro et al., 2014) and *H19* (Koukoura et al., 2011b), *PEG10* (Diplas et al., 2009) but also the downregulation of others, for example the *IGF2* gene (Cordeiro et al., 2014; Demetriou et al., 2014; Piyasena et al., 2015), and *MEST* (McMinn et al., 2006).

The genes *CDKN1C, H19, IGF2, KCNQ1* and *PHLDA2* are located in an imprinted gene cluster on human chromosome 11p15. This region harbours two imprinted domains regulated by DMRs. The DMR at the *H19/IGF-2* is also called Imprinted Control Region (ICR1) and the other DMR, at the *KCNQ1/CDKN1C*, is called ICR2. As the set of imprinted genes present in this region is involved in fetal growth, genetic and epigenetic mutations can lead to disturbance of DMRs, resulting in fetal growth disorders (Netchine et al., 2012; Schreiner et al., 2014) (Fig.9).

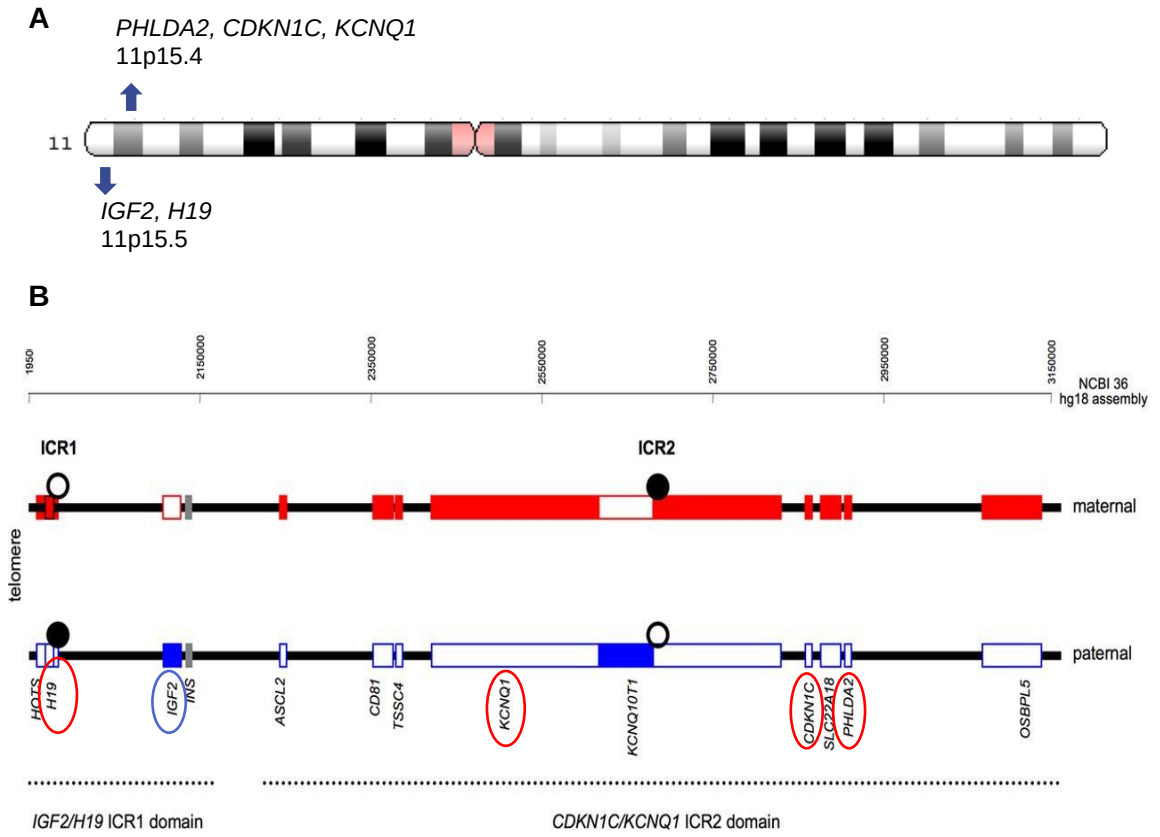


Figure 9 A- Chromosome 11 schematic diagram. *CDKN1C*, *PHLDA2* and *KCNQ1* are located on 11p15.4 and *IGF2* and *H19* are located on 11p15.5. Adapted from Genome Decoration Page/NCBI. **B-** Schematic illustration of genes located on 11p15 chromosome. This region harbours the ICR1 (regulates imprinted genes, such as *H19* and *IGF2*) and ICR2 (regulates imprinted genes, such as *KCNQ1*, *CDKN1C* and *PHLDA2*). The bold circles represent methylated ICR. The colored rectangles represent expressed maternal and paternal genes, red and blue respectively (Shmela and Gicquel, 2013). ICR- Imprinting control region.

a. Pleckstrin homology-like domain family amember 2 (*PHLDA2*) gene

The maternally expressed imprinted gene pleckstrin homology-like domain family amember 2 (*PHLDA2*) is highly expressed in placenta and encodes a protein with a Pleckstrin-homology domain, where phosphatidylinositol lipids can bind. Studies suggest that this type of proteins are associated with cell signalling, intracellular trafficking and membrane-cytoskeletal interactions, and also with growth suppression (Jensen et al., 2014). The control of this gene, *KCNQ1* gene and *CDKN1C* gene is made by the centromeric imprinting control region 2 (ICR2 or KvDMR1). In this region is also encoded

the promoter of an antisense ncRNA KCNQ1OT1 that interferes in the regulation of imprinting (Fig.10). Most studies suggest that *PHLDA2* gene expression is increased in fetal growth restriction cases or showed a negative correlation between birth weight and gene expression (Apostolidou et al., 2007; Diplas et al., 2009; Cordeiro et al., 2014; Shi et al., 2014). These results are in agreement with the conflict theory, indicating that maternally expressed gene *PHLDA2* limits resource provision (Frost and Moore, 2010). *PHLDA2* gene has an important role in nutrient exchange and increased gene expression results in fetal growth restriction, affecting the fetus and modifying the placenta (Frost and Moore, 2010; Moore et al., 2015).

Several studies made in mice confirmed the association between *Phlda2* expression and placental and fetal growth. It was demonstrated that *Phlda2* gene plays a role in fetal and placental development. In *Phlda2* knockout mice were reported an enlarged placenta and an increase in placental weight (Tunster et al., 2014, 2016). In addition to the relation between *Phlda2* and placenta weight, it was described IUGR in transgenic mice without KvDMR1. The absence of KvDMR1 leads to loss of imprinting, resulting in overexpression of *Phlda2* (Salas et al., 2004). As in the case of humans, mice placenta show modifications associated with the increase of *Phlda2* expression, in which occurs the reduction of spongiotrophoblast, leading to placental glycogen reduction that may affect fetal growth (Tunster et al., 2016). Although the differences of human placenta and mouse placenta, it also contains glycogen, that it is stored in extravillous cytotrophoblast (Jensen et al., 2014). In both mice and human, the intrauterine growth might be regulated by the role of *PHLDA2* in nutrient transfer.

b. Cyclin-dependent kinase Inhibitor 1C (*CDKN1C*) gene

The Cyclin-dependent kinase Inhibitor 1C (*CDKN1C*) is a gene that encodes a protein with an essential role in inhibiting several cyclin-dependent kinases (CDKs) with roles in G1/S-phase transition. The CDKs inhibition, by CDKN1C, results in inhibition of cell cycle progression, thus CDKN1C is considered a negative regulator of cell proliferation and a putative tumour suppressor (Lee et al., 1995).

The *CDKN1C* gene has four exons, in which the protein is coded by the exon 2 and the exon 3. The functional protein contains three keys domains, the N-terminal CDK inhibitory domain, the proline-alanine repeat (PAPA) domain and the proliferating cell nuclear antigen (PCNA) binding domain, that avoid DNA replication. In the mice CDKN1C protein, the PCNA binding domain is not present and the PAPA repeat domain is replaced by a proline-rich and acidic domain (López-Abad et al., 2016).

Studies demonstrate that *CDKN1C* mRNA transcripts are found in several tissues, such as testis, brain, lung, kidney, pancreas and skeletal muscle, however, this transcript is most abundant in placenta (Sood et al., 2006).

The deregulation of the maternally expressed *CDKN1C* gene is associated with growth disorders, such as the overgrowth disorder Beckwith-Wiedemann syndrome (BWS) and the IMAge syndrome (Eggermann et al., 2014; López-Abad et al., 2016).

Loss-of-function mutations are linked with the overgrowth disorder Beckwith-Wiedemann syndrome. On the other hand, the gain-of-function mutations within proliferating cell nuclear antigen (PCNA) domain is linked to disorders characterised by growth failure, namely the IMAge syndrome and Silver-Russel syndrome (SRS) (Arboleda et al., 2012; López-Abad et al., 2016).

In humans, it has reported the consistent upregulation of this gene in IUGR cases (McMinn et al., 2006; Cordeiro et al., 2014; López-Abad et al., 2016). The *CDKN1C* upregulation is associated with abnormal ICR2 methylation, truncating mutations and deletion of *KCNQ1OT1*, that result in increased expression of *CDKN1C*. In addition to that, the action of cis regulatory elements may also regulate the *CDKN1C* expression (Shmela and Gicquel, 2013).

In mice, the *Cdkn1c* gene is located on chromosome 7 and the expression is maternal. In 2011, Tunster and his collaborators concluded that *Cdkn1c* is involved in the allocation of maternal nutrients from the mother to the fetus, through the placenta. In this studies, the embryonic overgrowth was related to the absence of *Cdkn1c*, in knockout mice. Lastly, the observed overgrowth was attenuated due to the intrauterine competition (Tunster et al., 2011). Other studies also reported the association between increased *Cdkn1c* expression and embryonic growth restriction (Andrews et al., 2007; McNamara et al., 2016).

c. Potassium voltage-gated channel subfamily Q member 1 gene (*KCNQ1)*

The potassium voltage-gated channel subfamily Q member 1 gene, known as *KCNQ1*, is maternally expressed and controlled by the centromeric imprinting control region 2 (ICR2 or KvDMR1). Besides the controller function, KvDMR1 is also the promoter for the *KCNQ1* overlapping transcript 1 (KCNQ1OT1) ncRNA (Monk et al., 2006). On the paternal chromosome, the transcript silences the flanking imprinting genes, and on the maternal chromosome, the ICR2 is methylated and *KCNQ1OT1* is not transcribed, resulting in expression of flanking genes (Chiesa et al., 2012) (Fig.10).

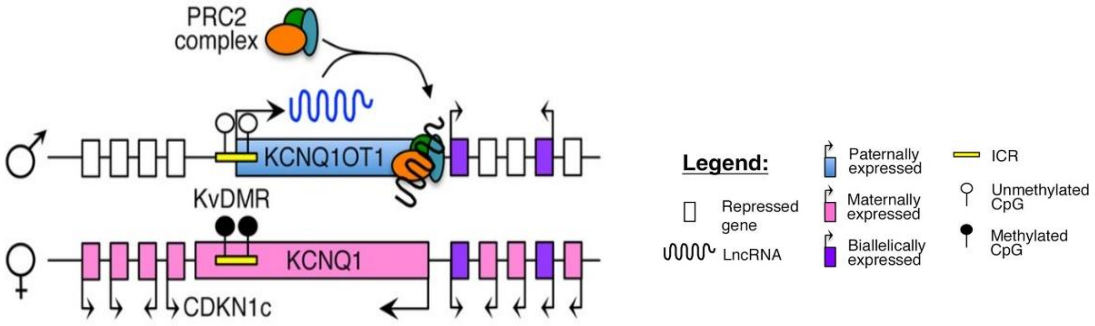


Figure 10 Model of imprinting at the *KCNQ1* locus. The paternal expressed KCNQ1 overlapping transcript 1 (KCNQ1OT1) silences the flanking imprinting genes (such *PHLDA2*, *CDKN1C*, *KCNQ1*) in the paternal allele. In the maternal allele, the ICR2 is methylated, the KCNQ1OT1 is silenced and the genes are expressed. PRC2- Polycomb repressive complex 2; ICR-Imprinting control region; LncRNA-Long non coding RNA (Kameswaran and Kaestner, 2014).

d. Insulin-like growth factor 2 *(IGF2*) and *H19* genes

The Insulin-like growth factor 2 *(IGF2*) and *H19* genes are located alongside to each other and their imprinting is controlled by methylation of imprinting control region (ICR1). *IGF2* is a paternally expressed gene and *H19* is a maternally expressed gene (Moore et al., 2015). The function of the first gene is to promote the growth and proliferation of cells and it is essential before birth. The second, it is a noncoding transcript and appears to play an important role in early development (Bergman et al., 2013). Hypomethylation of CpG sites on ICR1 decreases *IGF2* expression resulting in fetal growth restriction, while hypermethylation has the opposite effect (Hillman et al., 2015). ICR1 contains binding sites for CCCTC factor (CTCF) and *IGF2* gene expression occurs when DNA methylation at ICR1 on the paternal allele prevents the binding of CTCF. In contrast, *H19* expression results from CTCF binding to unmethylated maternal ICR1, acting as an insulator. In addition to that region also exist two regions of allele-specific methylation within the human *IGF2* (Piyasena et al., 2015) (Fig.11).

In mice, the *Igf2* gene is located on chromosome 7 and the complete loss of *Igf2* in placenta result in severe growth restriction of fetus and placenta (Constância et al., 2002; Fowden et al., 2006). The size reduction of placenta was observed in all placental layers, leading to abnormal nutrients exchange and IUGR (Sibley et al., 2004). In human IUGR studies is also observed the downregulation of this paternally expressed gene (McMinn et al., 2006; Cordeiro et al., 2014) and the same is observed in cases of SRS. In contrast, the *IGF2* upregulation is related to other disorders, such as BWS and tumour predisposition (Ishida and Moore, 2013).

Despite the colocalization with *IGF2* in the ICR1, *H19* is an imprinted maternally expressed gene that in IUGR is commonly upregulated. The *H19* upregulation, due to methylation alterations, is related to loss of imprinting and leads to restriction of growth.

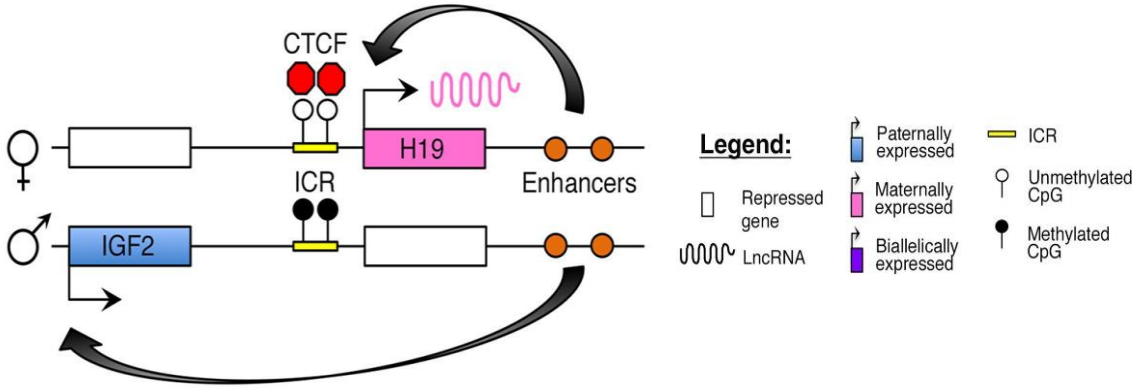


Figure 11 Model of imprinting at the *H19–IGF2* locus. DNA methylation *at* ICR1 on the paternal allele prevents CTCF binds, resulting in the expression of IGF2 and *IGF2* silence. In maternal allele, ICR1 is unmethylated and CTCF binds, resulting in the *H19* expression and *IGF2* silence. CTCF- CCTC factor; ICR-Imprinting control region; LncRNA- Long non coding RNA (Kameswaran and Kaestner, 2014).

Imprinted genes located on human chromosome 7 are also associated with fetal growth, such as paternal expressed gene 10 (*PEG10*) and mesoderm-specific transcript *(MEST) (*Fig.12) (Maccani and Marsit, 2009).

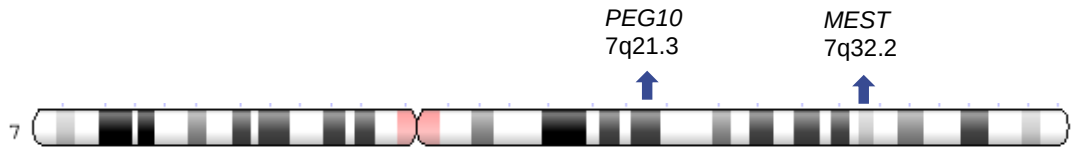


Figure 12 Chromosome 7 schematic diagram. *PEG10* is located on 7q21.3 and *MEST* is located on 7q32.2. Adapted from Genome Decoration Page/NCBI.

e. Paternal expressed gene 10 (*PEG10)*

PEG10 is located on human chromosome 7q21 and have evolved from retrotransposons (Ono et al., 2001). This gene exhibit a crucial role in mid-gestation placental function, acting as a regulator (Koppes et al., 2015). Chen and collaborators showed that trophoblast proliferation, differentiation and invasion are affected by silencing of *PEG10*. Moreover, studies demonstrated altered *PEG10* gene expression in some human pregnancy complications, like IUGR (Diplas et al., 2009).

In 2006, Ono reported early embryonic lethality in *Peg10* knockout mice due to limitations in the placenta, demonstrating the association of *Peg10* gene with placenta formation and development (Ono et al., 2006).

Although this gene is expressed paternally, several studies have shown that *PEG10* expression was increased in cases of growth restriction. Those results suggest that these

imprinted gene was acting in a compensatory manner (Diplas et al., 2009; Piedrahita, 2011).

f. Mesoderm specific transcript *(MEST) or* Paternal expressed gene 1 (PEG1) genes

The first imprinted gene mapped to chromosome 7 is *MEST*, also referred as Paternal expressed gene 1 (PEG1) (Kobayashi et al., 1997). This paternally expressed gene is located at 7q31-34 and encodes an α/β hydrolase fold family enzyme (Huntriss et al., 2013). In humans, *MEST* is expressed and imprinted in placenta and it is thought to have a role in angiogenesis, in trophoblast tissue and decidua (Frost and Moore, 2010). In addition, *MEST* expression was associated with a slight decrease in *MEST* methylation (Katari et al., 2009). In mice, *Mest* is located on chromosome 6 and the gene knockout resulted in fetal growth restriction (Moore et al., 2015).

II. Aims of the study

The main aim of this study is to find biomarkers that allow the identification of pregnancies with a higher risk of developping IUGR, namely severe IUGR. This could contribute to a better knowledge of intrauterine growth and leading at the end to *in utero* therapeutic options.

1. Imprinted genes effects on fetal growth, analyzing gene expression in normal and IUGR placentas.
2. DNA Methylation and DNA hydroxymethylation effects on fetal growth, studying 5-mC and 5-hmC levels in candidate genes in normal and intrauterine growth restriction pregnancies.
3. The relationship among 5-mC, 5-hmC, gene expression and intrauterine growth restriction.

III. Material and methods

1. Placental sample collection and storage

Placental tissue samples were collected from eleven normal pregnancies and twenty two IUGR pregnancies, at term (37 weeks to 40 weeks), by the obstetricians of the Gynecology and Obstetric Department, in Centro Hospital de São João, Porto. The IUGR was classified by an obstetrician and the criteria took into account biometrical parameters of the fetus below percentile 10 for gestational age, fetal anatomy and placenta evaluation (Campos et al., 2008). Only cases with normal karyotype were used in the present study. The donation of this tissue was accepted by the Health Ethics Committee of the Hospital/Faculty, and an informed consent was assigned and obtained from the mothers.

After sampling, segments were drawn from each placenta and wash two times in 1mL PBS 1x, removing the excess of blood. To avoid RNA degradation, 500mL of RNAlater was added to one tube of 1,5mL with one placental fragment. The placental samples were stored at -80ºC until RNA and DNA extraction.

2. Placental RNA and DNA Extraction

The frozen tissue was thawed on ice and washed with PBS 1x to remove RNAlater excess. After wash, the sample was transferred to a Triple-Pure™ zirconium beads tube (Benchmark Scientific) and 1mL of TRIzol reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA) was added to the tube. This reagent inhibited RNase activity, maintaining the integrity of the RNA during tissue homogenization. In addition to RNA extraction, the use of TRIzol allows the simultaneous precipitation of high-quality DNA and protein. The placental tissue was homogenized by a Minilys homogenizer (Peqlab) for 60 seconds at full speed, transferred to a 1,5mL tube and incubated at room temperature for 5 minutes. This step allows the complete dissociation of nucleoprotein complexes.

a. RNA isolation

Subsequently, it was added 200µL of chloroform to the dissociated tissue, which was shaken for 15 seconds and incubated at room temperature for 3 minutes, followed by a centrifugation at 12,000 x g for 15 minutes at 4ºC. After centrifugation, the samples were separated into three phases: a colourless upper phase (containing the RNA), a white interface (containing the DNA) and a lower red phase (containing the protein fraction).

RNA precipitation stage was initiated with the transfer of the colourless upper phase to 1,5mL RNase-free tube (the other phases were saved for further DNA extraction) and the addition of 500µl of isopropanol, incubating at room temperature for 10 minutes after

mixing by inversion. Subsequently to a centrifugation (12,000 x g for 10 minutes at 4ºC), the supernatant was discard and the RNA pellet was washed with 1mL of 75% (v/v) ethanol, followed by a centrifugation at 7,500 x g for 5 minutes,4ºC. RNA pellets were air dried for about 15 minutes at room temperature, after the supernatants were discarded. RNA pellet was resuspended in 20-50µL of RNase-free water (Qiagen, Germany) (according to pellet size) and incubated 10 minutes at 60ºC in Biotron Biometra TRIO Thermoblock Heat Cycler. RNA concentration and the purity was determined by NanoDrop 2,000 UV-vis Spectrophotometer (Nanodrop Technologies, Wilmington, USA). This procedure allows the subsequent use of an adequate amount of RNA, from 1µg to 10pg total RNA, to cDNA synthesis. In addition to concentration and purity analysis, a 0,8% gel electrophoresis in 0.5 × TBE was performed to evaluate RNA degradation. The extracted RNA was stored at -80ºC until further use.

b. DNA Isolation

For the isolation of DNA, it was added 300µL of 100% ethanol to the white interface and lower red phases. After inversion and incubation (3 minutes at room temperature), the samples were centrifuged at 5,000 x g for 5 minutes, 4ºC. The supernatant, which contained the protein, was removed and stored at -80ºC. The DNA pellet was washed with 1mL of 10% EtOH/0.1M sodium citrate pH 8,5 and incubated at 4ºC overnight. On the following day, the samples were centrifuged at 5,000 x g for 5 minutes,4ºC and the wash were repeated, but with an incubation at room temperature for 30 minutes. After the wash and centrifugation (5000 x g for 5 minutes,4ºC) the supernatant was removed and 1,5mL of 75% ethanol was added to each sample, incubating at room temperature for 20 minutes. After a brief centrifugation at 5,000 x g for 5 minutes at 4ºC, the ethanol was discarded and the pellets were air-dried for 5-10 minutes. The DNA pellets were resuspended in 200µL of 8mM NaOH and incubated at 37ºC for 1hour and at 4ºC overnight. After a centrifugation 12,000 x g for 10 minutes,4ºC and the supernatant transfer to a new tube, the DNA concentration and purity was determined by NanoDrop 2,000 UV-vis Spectrophotometer (Nanodrop Technologies, Wilmington, USA). For methylation and hydroxymethylation studies, the recommended amount of DNA is 1–10µg and 25-200 ng, respectively. The extracted DNA was stored at -20ºC until further use.

3. DNase Treatment

Total RNA extracted was treated with DNase I (Thermo Scientific™), before cDNA synthesis. This procedure is necessary to remove the genomic DNA from RNA samples. DNase I is an endonuclease that digests DNA.

Briefly, 1µg of each total RNA was added to the mix containing: 1µL of 10X reaction buffer with $MgCl_2$, 1µL (1U) of DNase I and diethylpyrocarbonate (DEPC)-treated Water (to obtain a final volume of 10 µL).
Samples were then incubated for 30 min at 37ºC, performed in a Veriti 96-Well Thermal Cycler (Applied Biosystems), and 1µL of 50mM Ethylenediamine tetraacetic acid (EDTA) was added to each sample. The enzyme deactivation was accomplished after an incubation for 10 minutes at 65ºC.

4. cDNA synthesis

Reverse transcription was executed in 1µg of total RNA (DNase treated) for cDNA synthesis, using qScript™ cDNA SuperMix (Quanta Biosciences, Inc., Gaithersburg, USA). cDNA was necessary to perform the Real-Time PCR and to analyze genes expression.
First, for each cDNA synthesis reaction was added the total volume of DNase treated RNA (11µL), 4µL of 5X qScript supermix, and 4µL of nuclease-free water, to a 0.2mL microtube. The contents were briefly mixed and centrifuged, and placed in the thermocycler. The samples incubation condition was 25ºC for 5 minutes, followed by 42ºC for 30 minutes, and finally 85ºC for 5 minutes. All samples were stored at -20ºC until further use.

5. Quantitative Real-Time PCR for gene expression analysis in normal and IUGR pregnancies

One of the uses of Real-Time PCR is for quantitative mRNA expression studies. This technique allows the monitorization of PCR reaction in real time and the quantification of PCR products. For that purpose, the PCR products are labelled with a reporter molecule, emitting fluorescence. The fluorescence intensifies with the increase of the PCR products. The reporter molecule used was a DNA-binding dye, the green fluorescent EvaGreen dye. Generally, the EvaGreen dye is nonfluorescent but becomes highly fluorescent after the binding to dsDNA (Fig.13).

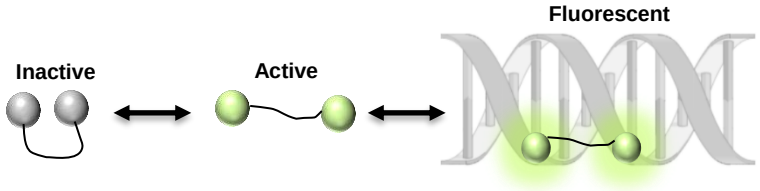


Figure 13 EvaGreen dye binds to dsDNA and becomes highly fluorescent.

In this study, RNA expression levels of imprinted genes (*IGF2*, *H19*, *CDKN1C*, *KCNQ1*, *PHLDA2*, *PEG10* and *MEST*) were assessed by Real-Time PCR on a StepOnePlus™ Real-Time PCR System (Life Technologies Corporation, California, USA). In addition to imprinted genes, housekeeping genes (*RPLP, TBP*) were analyzed for endogenous control.

In this procedure, 5x HOT FIREPol® EvaGreen® qPCR Supermix (Solis Biodyne, Tartu, Estonia) and specific primers for each imprinted gene (table 1) and housekeeping genes (table 2) were used. qRT-PCR was performed for each gene and included negative controls to detect any contamination. The samples were run in duplicate, to minimize intra-plate variations. In each single reaction, 13µL of RNase-free water, 4 µl of 5x HOT FIREPol® EvaGreen® qPCR Supermix, 0,5µl of each primer (20µM) (Metabion, Germany) and 2µL of diluted cDNA (1:10) were added. PCR parameters were as follow: 95 °C for 15 min, followed by 40 cycles at 95 °C for 15s, 60°C for 20 s and the final step was 72ºC for 30s.

Table 1 Primers of imprinted genes for qRT-PCR.

Gene	Sequence	Amplicon (bp)	Reference
CDKN1C	Fw 5' CGGCGATCAAGAAGCTGTCC 3'	186	Design in PrimerBlast
	Rv 5' TGGGCTCTAAATTGGCTCACC 3'		
PHLDA2	Fw 5' GCGACAGCCTCTTCCAGCTAT3'	178	Design in PrimerBlast
	Rv 5' TCGGTGGTGACGATGGTGAAGT 3'		
H19	Fw 5' GGAGTTGTGGGAGACGGCCTTGAGT 3'	111	Moore G. et al. 2015
	Rv 5' CCAGTCACCCGGCCCAGATGGAG 3'		
IGF2	Fw 5' ATGGGGAAGTCGATGCTGGT 3'	154	Design in PrimerBlast
	Rv 5' CGGGCCTGCTGAAGTAGAA 3'		
KCNQ1	Fw 5' TCGTTTACCACTTCGCCGTCT 3'	128	Design in PrimerBlast
	Rv 5' CACCACCAGCACGATCTCCATC 3'		
MEST	Fw 5' AGCTCTTGCCTCTGTAACTATCCC 3'	104	Design in PrimerBlast
	Rv 5' GCGGCAGCGTTTTCCTGTA 3'		
PEG10	Fw 5' CTGAGGAGAACAGCGGAGAAGG 3'	170	Design in PrimerBlast
	Rv 5' CGCTTATTTCACGCGAGGAC 3'		

Table 2 Primers of housekeeping genes for qRT-PCR.

Gene	Sequence	Amplicon (bp)	Reference
RPL0	Fw 5' GGCGACCTGGAAGTCCAACT 3'	149	Marques CJ. et al. 2011
	Rv 5' CCATCAGCACCACAGCCTTC 3'		
TBP	Fw 5' TGCACAGGAGCCAAGAGTGAAGA 3'	174	Design in PrimerBlast
	Rv 5' TTGGTGGGTGAGCACAAGGC 3'		

Before performing the qRT-PCR for each sample, a standard curve was constructed to calculate the gene-specific PCR efficiencies for each pair of primers. The standard curves were based on cDNA sample diluted in steps of ten-fold over three dilution points. The qRT-PCR mix and parameters were the mentioned above.

The standard curve was constructed for each gene and some parameters were determined, such as the correlation coefficients (R^2), slope values and the efficiency (E) of PCR. The last was calculated according to the equation E = 10^(-1/slope)-1.

6. *DNMT* and *TET* expression analysis using quantitative Real-Time PCR

The addition of a methyl group to cytosine of the CpG dinucleotides is achieved by the activity of DNMTs. On the other hand, TET enzymes catalyze the conversion of 5-mC to 5-hmC. Therefore, the analysis of *DNMT* and *TET* expression helps to analyze and understand DNA methylation and DNA hydroxymethylation levels.

Real-Time PCR was performed for *DNMTs* and *TETs* expression levels analysis, on a StepOnePlus™ Real-Time PCR System (Life Technologies Corporation, California, USA). The procedure is similar to method 5, however, the specific primers are for *TET1, TET2, TET3, DNMT1* and *DNMT3A* genes (Table 3).

Table 3 Primers of methylation genes and hydroxymethylation genes for qRT-PCR.

Gene	Sequence	Amplicon (bp)	Reference
TET1	Fw 5' TGGAAAGAAGAGGGCTGCGATGA 3'	164	Design in PrimerBlast
	Rv 5' GCACGGTCTCAGTGTTACTCCCTAA 3'		
TET2	Fw 5'AAGGCTGAGGGACGAGAACGA 3'	115	Klug et al., 2013
	Rv 5' TGAGCCCATCTCCTGCTTCCA 3'		
TET3	Fw 5'CAAGGAGGTGGAAATAAAGGCTGGT 3´	191	Design in PrimerBlast
	Rv 5´CGGGCTCTCTAGCACCATTGAC 3'		
DNMT1	Fw 5' TGGACGACCCTGACCTCAAAT 3'	168	Marques CJ. et al. 2011
	Rv 5' TGCTTACAGTACACACTGAAGCAG 3'		
DNMT3A	Fw 5' TATTGATGAGCGCACAAGAGAGC 3'	111	Marques CJ. et al. 2011
	Rv 5' GGGTGTTCCAGGGTAACATTGAG 3'		

7. Bisulfite genomic sequencing for DNA methylation detection in KvDMR1

DNA methylation generally occurs in the position 5 of cytosines, located in CpG dinucleotides, and it is an epigenetic mechanism of gene expression control (Bianco-Miotto et al., 2016). Thus, it is possible to determine the 5-methylcytosine levels of each gene, in normal cases and in IUGR cases, using the bisulfite genomic sequencing. This technique determines the "fifth base of DNA" (5-mC) at a single base-pair resolution in a qualitative, quantitative and efficient way (Li and Tollefsbol, 2011).

For DNA methylation analysis of the KvDMR1 region, bisulfite genomic sequencing was performed in seven DNA samples (3 controls and 4 IUGR). The genes selected for this analysis were those that had an expression significantly different in IUGR placentas and are regulated by KvDMR1 (*PHLDA2* and *CDKN1C).* The sample selection was based on the results of gene expression. In the IUGR cases, the selected samples were those that showed a greater alteration of the gene expression. In the control samples, the method of selection was the opposite of that used for the IUGR samples, selecting the samples that showed a lower gene expression.

a. Bisulfite conversion

The extracted DNA from 7 samples was submitted to sodium bisulfite treatment, using EpiTect Bisulfite Kit (Qiagen, Hiden, Germany). In this procedure, unmethylated

cytosines were converted to uracil and 5-methylcytosines (5-mC) remain unaltered (Fig.14).

	Original sequence	After bisulfite treatment
Unmethylated DNA	N-C-G-N-C-G-N-C-G-N	N-U-G-N-U-G-N-U-G-N
Methylated DNA	N-**C**-G-N-**C**-G-N-**C**-G-N	N-**C**-G-N-**C**-G-N-**C**-G-N

Figure 14 Bisulfite conversion with EpiTecT Bisulfite kit (Qiagen, Hiden,Germany).

First, 1µg of extracted DNA was treated with 85µL of bisulfite mix, 35µL of DNA protected buffer and RNase-free water (up to 140µL of final volume) in a Veriti 96-Well Thermal Cycler (Applied Biosystems). The bisulfite conversion thermal cycler conditions were as follow:

Table 4 Bisulfite conversion thermal cycler conditions

Time	Temperature
5 minutes	95ºC
25 minutes	60ºC
5 minutes	95ºC
85 minutes	60ºC
5 minutes	95ºC
175 minutes	60ºC
Indefinite	20ºC

After the conversion, a cleanup of bisulfite converted DNA was executed. The complete bisulfite reactions were briefly centrifuged and transfer to 1,5mL tubes. 560µL of Buffer BL was added to each sample and, after mix and centrifuge, the mixtures were transferred to Epitect spin columns. The columns were centrifuged for 1 minute at >14,000g, before and after the addition of 500µL of Buffer BW to spin column. Subsequently, the DNA was desulphonated with the addition of 500µL of Buffer BD to each column and incubated for 15 minutes at room temperature. After other centrifugation (1 minute at >14,000g), the columns were washed twice with 500µL of BW buffer, transferred to a new 2ml tube and again centrifuged for 1min at >14,000g. The DNA was eluted from the column with 20µL EB buffer (centrifugation for 1 minute at 15000x g) after placed the columns to a new 1,5mL tube. The Bisulfite modified-DNAs were stored at -20ºC until further use.

b. PCR amplification of KvDMR1 region

After bisulfite modification, modified DNA was amplified by PCR for KvDMR1 region. The reaction mix contained 38,3µL of H_2O BBraun, 5µL of 1x buffer with 1.5mM $MgCl_2$, 2,4µL of 10µM dNTPs (Invitrogen, Carlsbad, CA), 1µL of each primer (25µM) (Metabion,

Germany) (Table 5), 0,3µL of HotStarTaq enzyme (5 U/µl, Qiagen, Hilden, Germany) and 2µL of Bisulfite modified-DNA. The PCR conditions were: Initial denaturation (95ºC for 15 min), followed by 40 cycles of, denaturation (1 min, 94ºC), primer annealing (1 min, 60ºC), strand elongation (1 min,72ºC) and a final extension (20 min, 72ºC) (Marques et al., 2011).

The amplifications of bisulfite modified-DNAs were confirmed in a 2,5% gel electrophoresis 0.5 × TBE.

Table 5 Primers for PCR amplification of bisulfite modified DNA.

Gene	Sequence	Amplicon (bp)	Reference
KvDMR1	Fw 5' TGTTTTTGTAGTTTATATGGAAGGGTTAA 3'	360	(Khoueiry et al., 2013)
	Rv 5' CTCACCCCTAAAAACTTAAAACCTC 3'		

c. Bisulfite modified-DNA purification

The PCR products were purified using Agencourt AMPure XP beads (Beckman Coulter, Krefeld, Germany). This step is necessary to remove the reagents added in the PCR reaction that can inhibit the cloning reaction. AMPure® XP solution contains paramagnetic beads that selectively bind to PCR amplicons. After washes and elution, the PCR products were separated from contaminants.

The AMPure® XP solution was gently mixed to resuspend beads before 50µL of the solution was added to each PCR product. Samples were mixed by inversion and briefly centrifuge, and place on 96S Super Magnet Plate (Alpaqua) for 2 minutes, to separate beads from solution. The cleared solution was aspirated (with the tubes in the magnetic plate) and 100µL of fresh 70% ethanol was added to each sample. After the aspiration of the 70% ethanol, the tubes were removed from the plate and the beads air dried for 5 minutes. The DNA elution was completed adding 35µL Elution Buffer to each tube, containing the beads. The tubes were placed on a magnetic plate for 1 minute and 20µL of the supernatant was transferred to a clean 1.5mL microcentrifuge tube. The purified PCR products were stored at -20ºC until further use.

d. Cloning procedure

The method subsequent to the PCR products purification was the cloning procedure, using TOPO TA cloning kit (Invitrogen), pCR™II-TOPO® (Invitrogen) and NZY5α Chemically Competent *Escherichia coli* cells (NZYTech). The cloning and sequencing of bisulfite converted DNA allows the detection of single molecules distribution of methylation patterns, providing a higher resolution than direct PCR product sequencing (Li and Tollefsbol, 2011). The pCR™II-TOPO® vector includes a gene that confers

resistance to the antibiotic ampicillin, allowing the selection of colonies that incorporate the plasmid, when the medium has ampicillin. In addition to the gene that confers resistance to the antibiotic, the vector contains the lacZ gene that codes for β–galactosidase, an enzyme that digests the synthetic analogue of lactose (X-Gal) allowing the Blue-white selection. Colonies produce a blue color when the lacZ gene is functional, that occurs because the fragment was not inserted in the vector. The white colonies are those with the insert and those that should be chosen (pCR™II-TOPO® map in appendix).

The first step of cloning was the addition of adenines residues to the 3' blunt-ends of DNA fragments, using Taq DNA polymerase. Tailing allows that the vector binds more efficiently to the 3' ends of the PCR fragment, making it suitable for TA cloning. For this purpose, a mix containing 0,5µL of 10xTaq Buffer with $(NH_4)_2SO_4$ (Thermo Scientific), 0,5µL of 6µM dATPs, 0,2µl of Taq DNA polymerase 5 U/µL (Thermo Scientific), 0,2µL of 25mM $MgCl_2$ (Thermo Scientific) and 3,5 µL of the PCR product, was incubated at 72ºC for 15 minutes.

The next step of the cloning was the binding of the KvDMR1 PCR product (with A-overhang) to the pCRII TOPO vector (Invitrogen), using the kit TOPO TA Cloning (Invitrogen). For the ligation step, 1µL of PCR amplicon was added to 1µL of the salt solution, 3,5µL of DNA-free water, and 0,5µL pCRII TOPO vector. This ligation mixture was incubated at room temperature for 15 minutes and then put on ice.

Subsequently to ligation, transformation and cloning were performed using NZY5α Chemically Competent *Escherichia coli* cells. First, 4µL of each TOPO cloning reaction products were added to 100µL of competent cells (previously thawed on ice) and mixed gently. After an incubation on ice for 30 minutes, the cells were heat-shocked with 40 seconds in a 42ºC water bath and placed on ice for 2 minutes. To grow the bacteria was added 400µL of SOC medium and incubated for 1 hour at 37ºC with shaking (225rpm). After bacteria growth, 100µL and 250µL of the cells transformed with the ligation reaction were spread in two different pre-warmed LB agar plates with ampicillin (50mg/mL, AppliChem) and 40 µL of X-Gal (40mg/mL, Sigma). After overnight growth of cultures at 37°C, 20 white clones were picked and mix with 100µL of TE (10^{-1}). These clones were also culture overnight (37ºC) on pre-warmed LB agar plate with ampicillin (50mg/mL, AppliChem) and 40µL of X-Gal (40mg/mL, Sigma).

e. Plasmid amplification

PCR was performed to analyze the clones that incorporated the desired fragment. A mix for each sample was prepared that contained, 28,8µL of H_2O BBraun, 5µL of 10xTaq Buffer with $(NH_4)_2SO_4$ (Thermo Scientific), 2 µL of 10µM dNTPs, 0,2µL of Taq DNA

polymerase 5 U/µL (Thermo Scientific), 3µL of 25mM $MgCl_2$ (Thermo Scientific), 0,5µL of primer M13 forward and reverse (25µM) and 10µL of bacteria resuspension. The PCR conditions were: 94ºC for 10minutos, 35 cycles: 94ºC for 45 seconds; 50ºC for 45 seconds and 72ºC for 1 minute and 72ºC for 10 minutes. After the amplification, the products were analyzed in a 2,5% electrophoresis gel in TBE 0,5% and 15 clones containing the fragment of interest were selected for further sequencing.

f. Purification and sequencing of clones

After the selection of 15 clones for each sample, the PCR products were purified with AMPure® XP solution, as mentioned before in Bisulfite modified-DNA purification. The clones containing the target DNA were sequenced by Big Dye terminator Cycle Sequencing V1.1 ready reaction Kit (Applied Biosystems, Foster City, CA, USA). For reaction sequencing with a final volume of 10µL was mixed 2-5µL of purified product, 1µL of Terminator Ready Reaction Mix (2,5X), 1µL of Sequencing buffer (5X), 0,5µL of primer M13 forward (10µM), and 5µL of H_2O BBraun. The reaction conditions are described in Table 6.

Table 6 Conditions of the sequencing reaction.

Time	Temperature	Cycles
3 minutes	94ºC	1
10 seconds	96ºC	24x
5 seconds	50ºC	
4 minutes	60ºC	1x
Indefinite	4ºC	1x

The following step was the products precipitation, using a similar technique to the purification, however, in the procedure was used Agencourt CleanSEQ beads (Beckman Coulter, Krefeld, Germany). The precipitation step is required to remove the reagents used for the sequencing reaction. Initially, 10µL Agencourt CleanSEQ was added and mixed with the product of each clone (of each sample). Next, 85% ethanol was added, mixed and the tubes were placed on 96S Super Magnet Plate (Alpaqua) for 5 minutes. After the beads separated from the solution, the solution was discard and 100µL of 85% ethanol was added and discard after 30 seconds. The ethanol wash was repeated and the beads were air dry for 6 minutes. The subsequent step was the product elution with 20µL of H_2O BBraun and further transfer (10µL) to sequencing plate, after 5 minutes of elution and 5 minutes on the paramagnetic plate.

The sequencing results were viewed and analyzed in 3500 Genetic Analyzer (Applied Biosystems).

8. ELISA assays for DNA hydroxymethylation detection and quantification

The 5-hmC content of the extracted DNA samples was measured in duplicate by a hydroxymethylated DNA quantification kit, the QUEST 5-hmC™ DNA ELISA kit (Zymo research, USA), following manufacturer's instructions.

This ELISA-based technique is subdivided into 5 main steps: Coating, Blocking, DNA binding, addition anti-DNA HRP Antibody and Color Development. Briefly, for coating, 100µL of diluted anti-5hmC Polyclonal Antibody was added to each well. The Blocking step was executed adding 200 µL 1x ELISA Buffer. The denatured DNA was diluted to a final concentration of 1ng/µL and added to each well, to bind the antibody. Subsequently, 100µL of diluted Anti-DNA HRP Antibody was mixed in each well and 100µL 1xDevelopper was added to allow colour detection. The colour development was measured after 33 minutes (Abs 405) on an ELISA plate reader (Sunrise, Tecan).

This procedure allowed the detection and quantification of 5hmC (in percentage), comparing the absorbance of each sample to controls and using a standard curve.

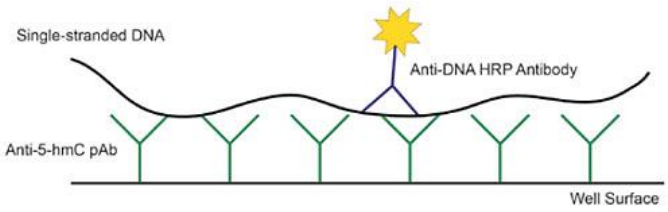


Figure 15 Overview of global 5-hmC detection via QUEST 5-hmC™ DNA ELISA.

9. Statistical analysis

The raw data obtained after qRT-PCR were introduced in qbasePlus (BioGazelle). This programme converts the quantification cycle values (Cq) to relative quantities (RQ). After a normalization and a calibration, the RQ values are converted in normalized relative quantities (NRQ) and then in calibrated normalized relative quantities (CNRQ). The software shows the expression results for each gene in CNRQ (Hellemans et al., 2007). In addition to that conversion, the obtained results were normalized with two reference genes (*RPL0* and *TBP*). The Mann-Whitney U test was used to compare the expression of genes between the two groups (IUGR vs Control). The relation between the imprinted genes was explored by Spearman's Correlation.

The bisulfite sequencing data were analysed in BiQ Analyzer (Bock et al., 2005). The comparison of the CpG methylation means between the two groups was evaluated with

a t-test, and the association between gene expression and methylation with Pearson correlation, in Statistical Package for Social Sciences (SPSS, IBM) software v24.0 database. The global hydroxymethylation data was evaluated using t-test in SPSS.
The results for all tests were considered significant when the p value was under 0,5.

IV. Results

1. Gene expression in IUGR placentas vs normal placentas

The levels of imprinted genes expression (*PHLDA2*, *CDKN1C*, *H19, IGF2*, *KCNQ1*, *PEG10* and *MEST*), methylation genes expression (*DNMT1* e *DNMT3A*), hydroxymethylation genes expression (*TET1*, *TET2* and *TET3*), and reference genes expression (*RPL0* and *TBP*) were accessed to evaluate the differences between IUGR cases and normal cases. The expression of the DNA methyltransferases, *DNMT3B* and *DNMT3L*, were not quantitatively assessed due to the low expression in the placental tissue.

qRT-PCR was the technique used to access the expression of all genes of interest and the software used to analyze the results was qBasePLUS (Hellemans et al., 2007).

Previously to gene expression analyses, standard curves for each gene were created and the efficiency of amplification was calculated using these standard curves. For this procedure, the same sample of a normal case was used for all genes. The results are included in the appendix.

The gene expression was determined for a total of 33 term placentas, of which 11 were from normal pregnancies and 22 were from pregnancies diagnosed with IUGR.

The raw data obtained after qRT-PCR were imported for software qBasePLUS, allowing the conversion of quantification cycle value (Cq) to quantities, by delta Cq method. The corrected values for amplification efficiencies were scaled to the group of samples of normal placentas and normalized with the two reference genes (*RPL0* and *TBP*). The results obtained for imprinted genes and genes related to methylation and hydroxymethylation are summarized in the Fig. 16.

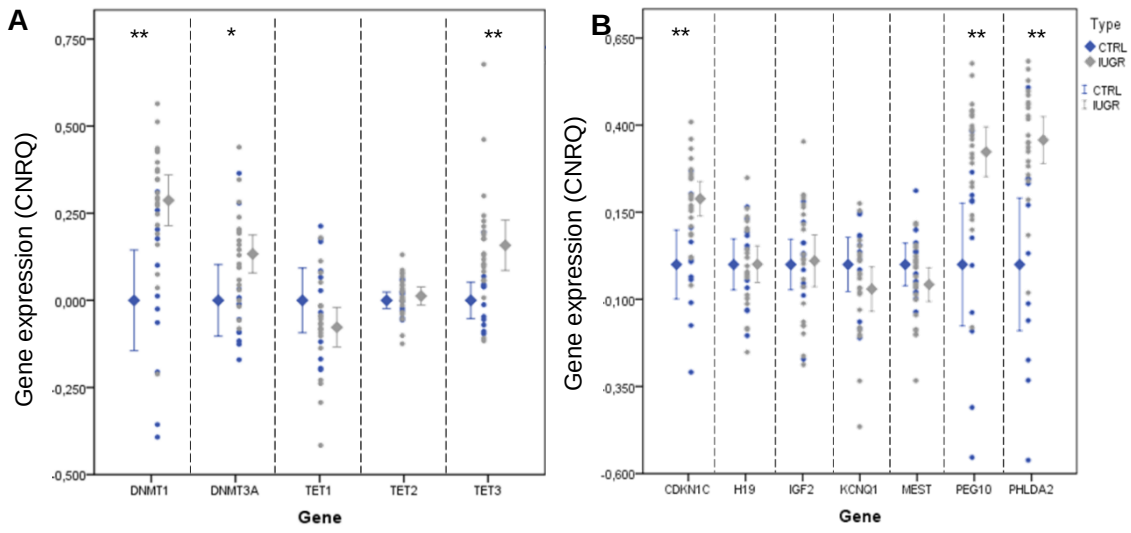


Figure 16 Gene expression graphs. *A- DNMT1, DNMT3A, TET1, TET2* and *TET3* expression in CNRQ (calibrated normalized relative quantities). *B-CDKN1C, H19, IGF2, KCNQ1, MEST, PEG10* and *PHLDA2* expression in CNRQ (calibrated normalized relative quantities). The dots represent the expression value for each sample and the diamond represent the mean expression for each gene with respectively error bar. * represent the p-value <0.05; ** represent the p-value<0,01; *** represent the p-value<0,001. CTRL- control samples; IUGR- Intrauterine growth restriction samples.

Mann-Whitney U statistical test was performed to evaluate the differences in expression in IUGR versus normal pregnancies. *PHLDA2*, *PEG10*, *CDKN1C*, *DNMT1*, *DNMT3A* and *TET3* and were significantly differentially expressed between the two groups (p value <0.05). The p values were 0,0023 for the *PEG10*, *PHLDA2*, *DNMT1* genes. The others two significantly differentially expressed genes, *TET3* and *CDKN1C*, exhibited a p value of 0,0044. Finally, the p value of *DNMT3A* was 0,019. In all significantly differentially expressed genes, the expression was significantly higher in IUGR cases than in normal cases. The other genes of interest exhibited expression values that are no significantly related with IUGR. Despite the non-significance of the expression values, the genes *KCNQ1*, *TET1* and *MEST* (p= 0,2458, p=0,3495 and p=0,3526, respectively) showed a tendency for downregulation in IUGR cases. The results are summarized in table 7.

Table 7 Gene expression value and p-value for each gene, values obtained comparing IUGR and Controls groups.

Gene	P value	Comparison ratio (IUGR vs Control)
TBP		0,912
RPL0		1,096
TET1	0,3495	0,837
TET2	0,4261	1,029
TET3	**0,0044**	**1,439**
DNMT1	**0,0023**	**1,806**
DNMT3A	**0,0190**	**1,359**
PHLDA2	**0,0023**	**2,276**
CDKN1C	**0,0044**	**1,544**
IGF2	0,9551	1,025
MEST	0,3526	0,876
PEG10	**0,0023**	**2,105**
H19	0,9551	1,002
KCNQ1	0,2458	0,8502

The association between all genes expression was analyzed by the Spearman's rank correlation coefficient. The results of this nonparametric test are in table 8.

	PHLDA2	CDKN1C	KCNQ1	IGF2	H19	MEST	PEG10	DNMT1	DNMT3A	TET1	TET2	TET3
PHLDA2	1,000											
CDKN1C	0,798***	1,000										
KCNQ1	-0,583**	-0,535**	1,000									
IGF2	-0,323	0,003	0,471*	1,000								
H19	-0,421*	-0,200	0,575**	0,705***	1,000							
MEST	-0,342	-0,475*	0,324	0,255	0,386*	1,000						
PEG10	0,877***	0,642***	-0,342	-0,221	-0,263	-0,273	1,000					
DNMT1	0,889***	0,690***	-0,612**	-0,386*	-0,476*	-0,392*	0,780***	1,000				
DNMT3A	0,641***	0,624***	-0,637**	-0,161	-0,435*	-0,266	0,447*	0,599**	1,000			
TET1	-0,501**	-0,658**	0,536**	0,266	0,409*	0,699***	-0,405*	-0,484*	-0,576*	1,000		
TET2	0,244	0,218	-0,083	0,100	0,053	0,031	0,338	0,094	0,353	-0,198	1,000	
TET3	0,408*	0,634***	-0,254	0,394*	0,057	-0,273	0,290	0,303	0,557*	-0,410*	0,484*	1,000

Table 8 Spearman's rank correlation coefficient between the gene expressions *- represent p-value<0.05; ** represent the p-value<0,01; *** represent the p-value<0,001.

The results for the seven imprinted genes showed a correlation between some of the genes localized in the same cluster. The genes *PHLDA2*, *CDKN1C*, *KCNQ1*, *IGF2* and *H19* are localized in the chromosome 11 at p15.5 and the results demonstrated the positive correlation between *PHLDA2* and *CDKN1C*, *KCNQ1* and *IGF2*, *KCNQ1* and *IGF2*, and finally *IGF2* and *H19*. In addition to the positive correlation between some genes, the results also showed a negative correlation between *PHLDA2* and *KCNQ1*, *PHLDA2* and *H19*, *CDKN1C* and *KCNQ1*.

The genes located at the chromosome 7, *MEST* and *PEG10*, are also correlated with some of the genes located in 11p15.5. *MEST* was positively correlated with H19 and negatively correlated with *CDKN1C*. On the other hand, *PEG10* was positively correlated with *PHLDA2* and *CDKN1C*.

In addition to the correlation results for imprinted genes, the correlation for DNMT's and *TETs* expressions was also evaluated. In the results of Spearman correlation was observed a positive correlation between the two *DNMTs*. The *TET3* expression was negatively correlated with *TET1* expression and positively correlated with *TET2* expression.

2. Methylation of KvDMR1 in placentas IUGR

A total of seven placental samples from fetuses diagnosed with IUGR (n=4) and normal placentas (n=3) were analyzed using the sequencing technique, after a bisulfite modification and cloning. The selected samples are Control 1:117198, Control 2:117200, Control 3: 117649, IUGR 1: 115521, IUGR 2: 115540, IUGR 3:116824 and IUGR 4: 117040 .

Among all genes studied, two genes regulated by ICR2 or KvDMR1 (*PHLDA2 and CDKN1C* genes) were significantly overexpressed in IUGR samples. Thus, the methylation of KvDMR1 was studied for the seven samples selected.

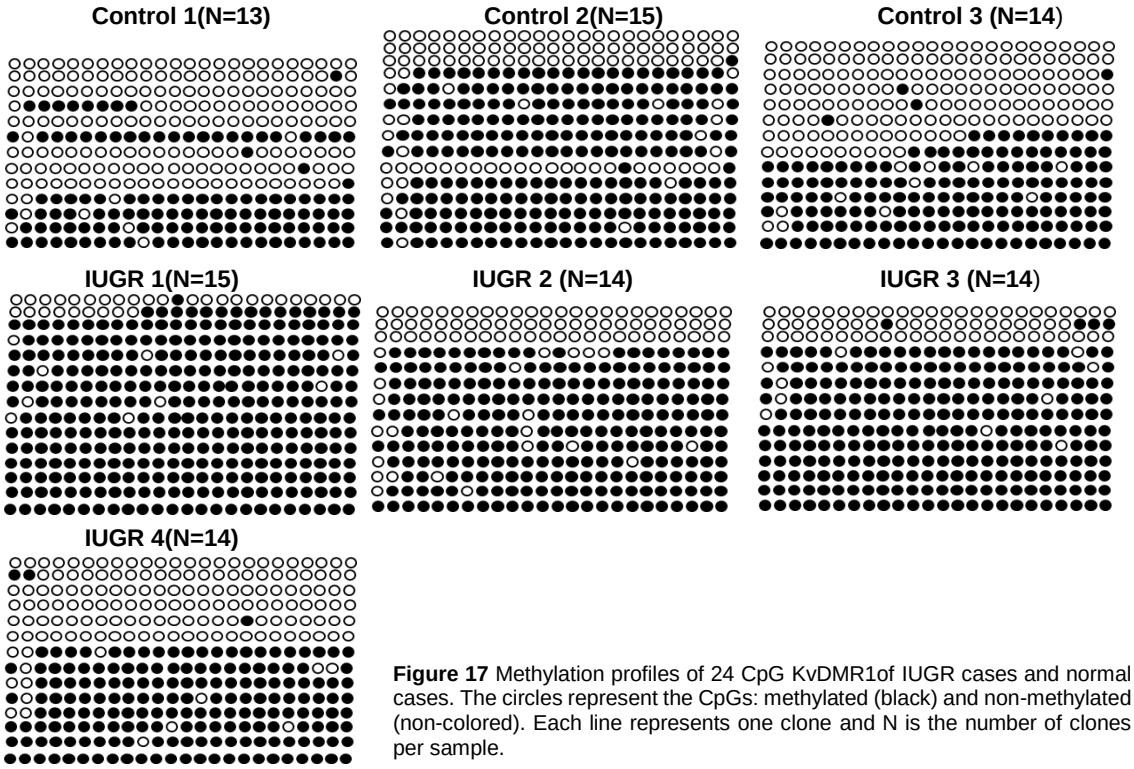


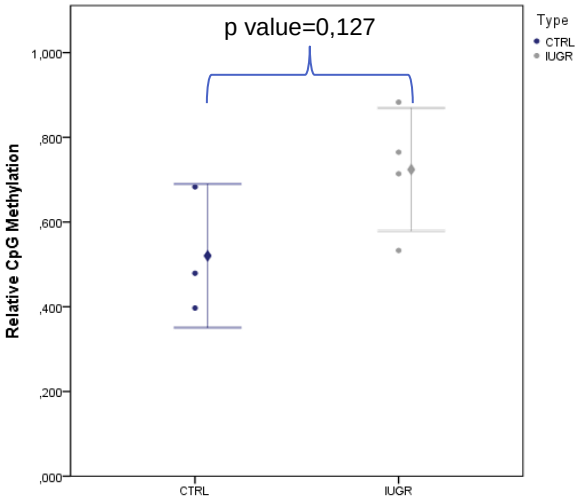
Figure 17 Methylation profiles of 24 CpG KvDMR1of IUGR cases and normal cases. The circles represent the CpGs: methylated (black) and non-methylated (non-colored). Each line represents one clone and N is the number of clones per sample.

The results analyzed by BiQanalyzer software demonstrated an increase in relative methylation in KvDMR1 of the IUGR placentas (Average percentage of methylation= 72,4) in relation to the control placentas (Average percentage of methylation= 52,0). Table 9 and figure 18.

Table 9 Relative methylated CpG of IUGR and normal cases. CTRL- control samples; IUGR- Intrauterine growth restriction samples.

Samples	Relative Methylated CpG
CTRL 1	0.397
CTRL 2	0.683
CTRL 3	0.479
Mean CTRL	**0,520**
IUGR1	0.883
IUGR 2	0.714
IUGR 3	0.765
IUGR 4	0.533
Mean IUGR	**0.724**

Figure 18 Comparison of relative methylated CpG between IUGR and normal cases. CTRL- control samples; IUGR- Intrauterine growth restriction samples.



However, the difference observed between the groups is not significant (p=0,127; p>0,005), as showed the t-test.

The association between gene expression (*CDKN1C* and *PHLDA2*) and relative methylated CpG in KvDMR1 was analyzed by the Pearson rank correlation coefficient. The results of this test are in table10 and in figure 19.

Table 10 Pearson correlation between gene expression (in CNRQ values) and relative methylated CpG of KvDMR1.

Genes	Pearson correlation	P Value
CDKN1C	0,613	0,143
PHLDA2	0,669	0,101

Despite the tendency for a positive association between genes expression (*CDKN1C* and *PHLDA2*), the results show that this positive association is non-significant.

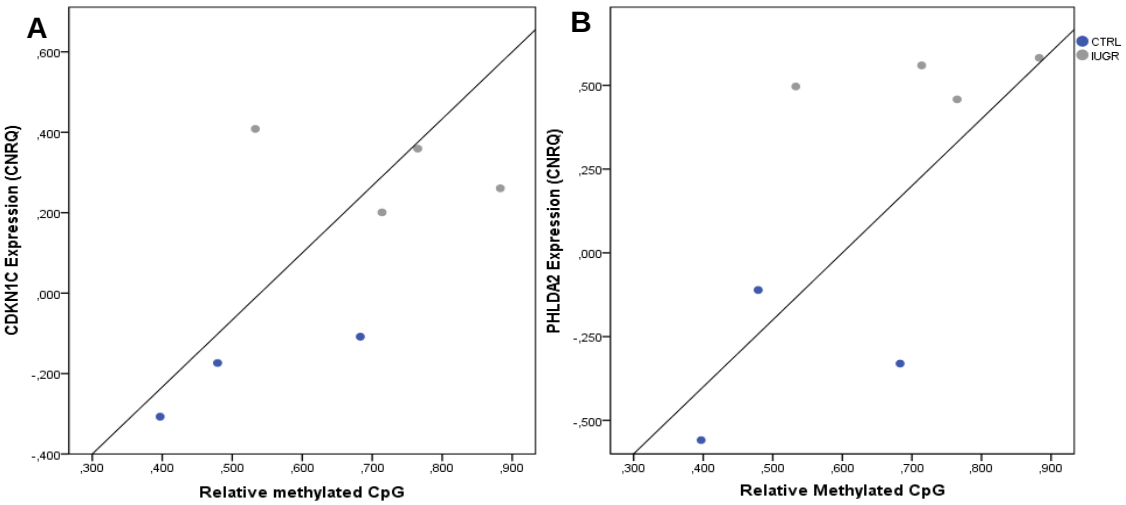


Figure 19 Correlation graph between gene expression and relative methylated CpG of KvDMR1. A- Correlation between *CDKN1C* expression and methylation. B- Correlation between *PHLDA2* expression and methylation. CTRL- control samples; IUGR- Intrauterine growth restriction samples; CNRQ- Calibrated normalized relative quantities.

3. Global Hydroxymethylation in placentas IUGR

The global hydroxymethylation for 33 DNA samples (22 IUGR samples and 11 normal samples) was assessed in duplicate by a colorimetric assay. In addition to this sample, 5 control samples were also assessed to construct a standard curve. The standard curve and respectively equation are shown in figure 20.

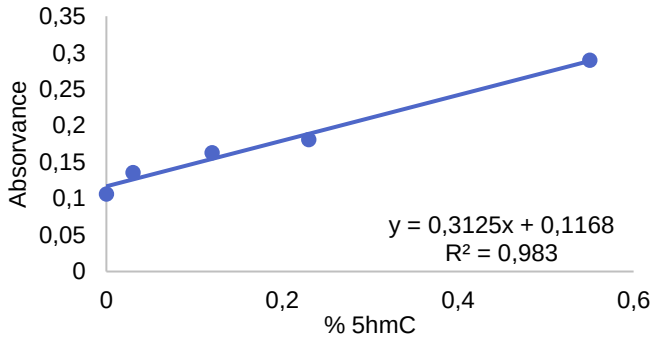


Figure 20 Standard curve for determination of global 5-hydroxymethylcytosine percentage.

The % 5hmC for each sample was calculated using the equation and the results are shown in figure 21.

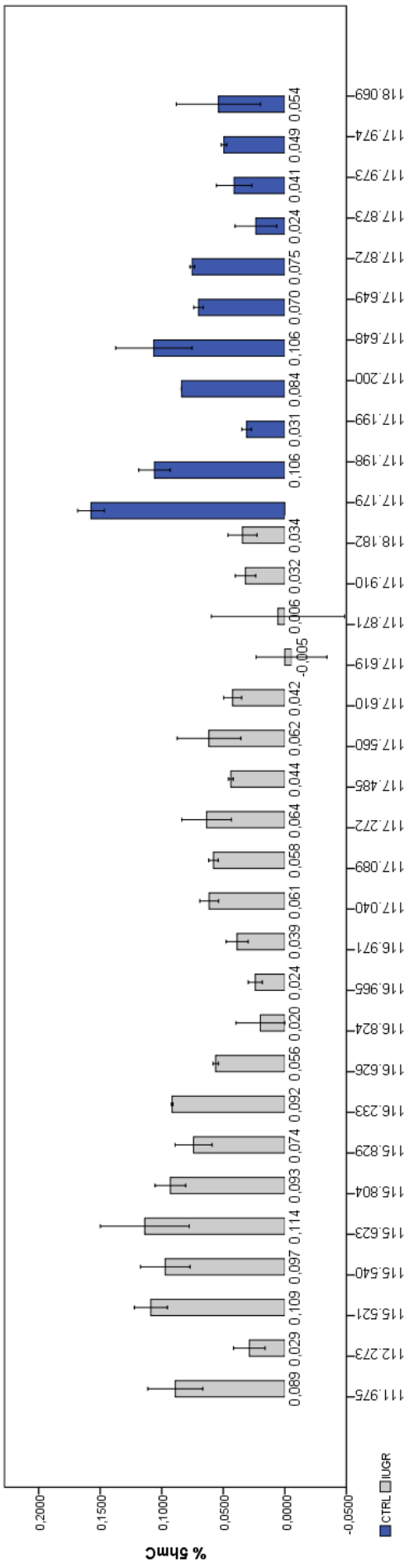


Figure 21 Percentage of 5-hmC (5 hydroxymethylcytosine) for all samples (22 IUGR and 11 controls). CTRL- control samples; IUGR- Intrauterine growth restriction samples.

The IUGR samples exhibited a lower percentage of global hydroxymethylation (mean=0,056%) than the normal samples (mean=0,073%). However, the results of t-test demonstrated that the decrease of global 5-hmC in IUGR samples is not significantly different from normal samples (p>0,05). The results are shown in figure 22.

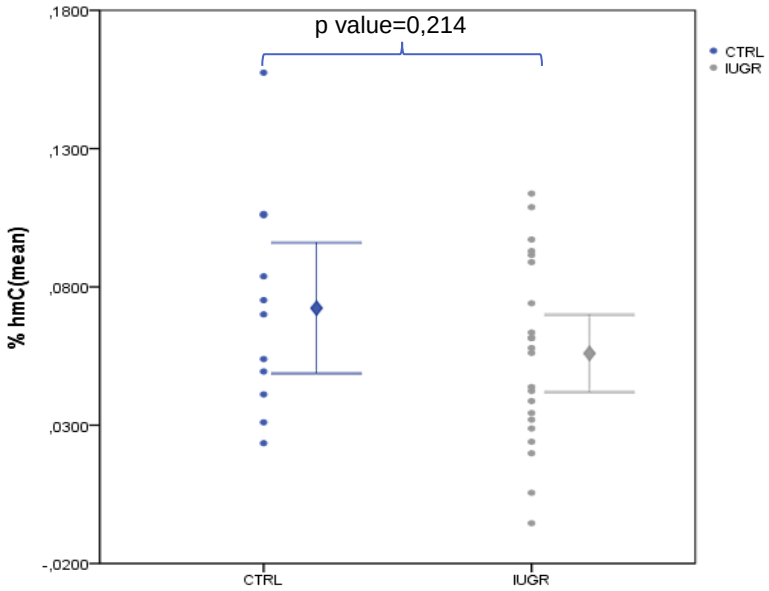


Figure 22 Comparison between the mean percentage of global 5hmC between the two groups (IUGR vs. Controls)**.** The dots represent % of global 5hmC for each sample and the diamond represent the mean with respectively error bar. * represent p-value <0.05. CTRL- control samples; IUGR- Intrauterine growth restriction samples.

V. Discussion

IUGR is a major cause of mortality and morbidity in fetus and neonates. It is estimated that this condition occurred in 5% to 7% of live births, 7% of stillbirths and 52% of unexplained stillbirths (Calkins and Devaskar, 2015). The IUGR study is of clinical relevance by virtue of IUGR high frequency and multifactoriality. The causes of IUGR lead to a change in fetal access to nutrients due to alteration in placental genes expression (Cordeiro et al., 2014). Several genes may be involved in IUGR aetiology, some of which are imprinted genes that play an important role in placental formation and development and fetal development (Ishida and Moore, 2013). Reports demonstrated that paternally expressed genes promote fetal growth and maternally expressed genes have the opposite effect (Moore et al., 2015). In addition to that evidence, other studies reported the association between imprinted gene expression with DNA methylation and, more recently, with DNA hydroxymethylation. Thus, it is important to find biomarkers that could allow the prediction of intrauterine growth restriction. To achieve this goal, the expression of imprinted genes (P*HLDA2, CDKN1C, H19, IGF2, KCNQ1, MEST* and *PEG10*), genes associated with methylation (*DNMT1* and *DNMT3A*), genes associated with hydroxymethylation (*TET1*, *TET2* and *TET3*) and reference genes (*RPL0* and *TBP*) were analyzed and compared in IUGR and normal placentas. These term placentas were also studied to evaluate and compare the DNA methylation of KvDMR1 and the global DNA hydroxymethylation levels. Until this date, several reports evaluated imprinted gene expression, but few studies compare the expression with DNA methylation levels (McMinn et al., 2006; Bourque et al., 2010; Koukoura et al., 2011a; Cordeiro et al., 2014; Iglesias-Platas et al., 2014; Jensen et al., 2014; López-Abad et al., 2016). The DNA hydroxymethylation is a recent concept whose study in human IUGR cases is not known. Recently, researchers observed the presence of that epigenetic modification in placental imprinted loci and they hypothesized that, in some previous studies, 5hmC may have been misinterpreted as 5mC (Piyasena et al., 2015).

The results from mRNA expression demonstrated that the levels of the imprinted genes, *PHLDA2*, *CDKN1C* and *PEG10*, were abnormally upregulated in the IUGR cases. However, the others imprinted genes showed non-significant differences between IUGR and normal samples. Of the seven imprinted genes studied, only three exhibited gene expression alterations in the IUGR cases (*CDKN1C*, *PHLDA2* and *PEG10*). The observed overexpression of the maternal expression genes, *CDKN1C* and *PHLDA2*, in IUGR samples, demonstrated that these results corroborate the imprinting conflict theory. However, for *PEG10*, a paternally expressed gene, the overexpression is not in agreement with the theory. According to imprinting conflict theory, the maternally expressed genes limit the nutrient supply, constraining the fetal growth. On the other hand, the paternally expressed genes influence the extraction of nutrients from the

mother, leading to fetal growth (Moore, 1991). However, this theory may not apply to the human as it applies in the mice since the human usually has singleton pregnancies and the absence of competition relieves the pressure for maintaining the imprinting (Diplas et al., 2009). By other side, some effects could be not causative of the pathology but arise to compensate the adverse effects of other genes.

The results obtained for *PHLDA2* gene are in agreement with several reports that demonstrated the overexpression of *PHLDA2* gene in placentas from pregnancies diagnosed with fetal growth restriction and with low birth weight (McMinn et al., 2006; Apostolidou et al., 2007; Diplas et al., 2009; Janssen et al., 2016). In mice, the *Phlda2* overexpression was also evaluated and associated with restriction of fetal growth, demonstrating that the increase of expression was a cause for FGR and suggesting that *Phlda2 gene* influence maternal-fetal transfer of nutrients (Tunster et al., 2010, 2016). Thus, the present study supports the association between IUGR and *PHLDA2* expression, highlighting the use of the *PHLDA2* gene as a possible growth restriction biomarker.

In addition to *PHLDA2* gene, *CDKN1C* gene was also significantly overexpressed in term placentas diagnosed with IUGR. This paternally imprinting gene is located alongside with the *PHLDA2* gene (11p15.5) and both genes are regulated by the same imprinting control region (ICR2 or KvDMR1) (Frost and Moore, 2010). *CDKN1C* gene encodes a protein that inhibits cyclin/Cdk complexes, which induces cell cycle arrest. IUGR placentas may be associated with abnormal cell proliferation and cell cycle alterations (Unek et al., 2014). Over the years some reports evaluated the relation between fetal growth and the *CDKN1C* gene in humans, demonstrating the overexpression of this gene in IUGR cases and in Small for Gestational Age (SGA) (McMinn et al., 2006; Piyasena et al., 2015; López-Abad et al., 2016). The *Cdkn1c* gene overexpression was also observed in growth restricted mice and the gene knockout or loss of function mutations enhanced cell differentiation and proliferation, leading to placental overgrowth (Andrews et al., 2007; Tunster et al., 2011). Our data, showing increased *CDKN1C* expression in IUGR placenta, are in consistent with the conflict theory and with some studies in human and mice. Thus, this maternally expressed gene may also have an important role in IUGR.

PEG10 gene, located on human chromosome 7q21, is derived from a retrotransposon (Ono et al., 2001). In contrast to *PHLDA2* and *CDKN1C* genes, *PEG10* is not in a gene cluster and is not regulated by an ICR. In mice, this paternally expressed gene showed to have a crucial role in placental development, intervening in the trophoblast proliferation (Chen et al., 2015). Thus, growth retardation in mice was associated to underexpression of *Peg10*, due to incomplete placenta formation (Ono et al., 2006; Bressan et al., 2009).

However, studies in humans have demonstrated the upregulation of this gene in cases of IUGR, indicating a possible compensatory response (Diplas et al., 2009; Piedrahita, 2011). Our results are in agreement with literature but are apparently in discordance with theory of parental conflict. The overexpression of the paternally expressed gene *PEG10* may be a response to fetal growth restriction, being a positive effector that act as a compensatory manner. However, more studies are needed to assess whether this is a compensatory mechanism. In future studies, an assessment of *PEG10* expression in placentas from different trimesters could be useful to understand if significant differences in expression occur over the three trimesters. Furthermore, that study would also elucidate at which stage begin the compensatory response. A preview study by our group, evaluated the expression of *PEG10* in the three trimesters, demonstrating no statistical significant differences in gene expression, however, the number of IUGR cases studied was limited (4 cases) (Dória et al., 2010).

PHLDA2, *CDKN1C* and *KCNQ1* gene are in the same cluster of genes and the three imprinted genes are regulated by the same imprinting control region, ICR2 or KvDMR1. The KvDMR1 is a region that controls the gene cluster and the long noncoding transcript KCNQOT1. The unmethylation of KCNQOT1 promotor allows the lncRNA transcription and this silences the cluster genes. On the other hand, the methylation of KvDMR1 blocks the KCNQOT1 transcription, leading to imprinted genes expression (Chiesa et al., 2012). The KvDMR1 ICR is usually methylated on the maternal allele, repressing expression of the lncRNA. On the paternal allele, KvDMR1 is unmethylated and promotes expression of KCNQ1OT (Diaz-Meyer, 2003).

Recently a report demonstrated the suppression of maternally expressed genes in mice with BWS, due to the absence of DNA methylation at KvDMR1, which led to the biallelic expression of Kcnq1ot1 (Singh et al., 2017). Other studies in mice also showed the biallelic expression of the transcript when a deletion of the promoter or a premature termination of the transcript was present (Jensen et al., 2014). In humans, the deletion of only ICR2 in the paternal allele leads to in silencing of KCNQ1OT1 and activation of *CDKN1C* and *PHLDA2*, causing severe IUGR (De Crescenzo et al., 2013). Human overgrowth disorder called Beckwith–Wiedemann syndrome is associated with loss of maternal methylation of ICR2 and this may cause fetal overgrowth, due to downregulation of maternally expressed gene (Beatty et al., 2006).

Our gene expression results showed the significant up-regulation of two genes regulated by the KvDMR1 (*PHLDA2* and *CDKN1C* genes*),* in IUGR cases. Thus, the evaluation of methylation status in the KvDMR1 is important for a better understanding of the disorder. The sequencing after bisulfite modification and cloning was performed for seven samples (3 control cases and 4 IUGR cases). The results showed no significant differences

between the two groups of samples. In addition to the non-significant difference between the 2 groups, in this study was also observed a tendency to a positive correlation between the genes expression (*CDKN1C* and *PHLDA2*) and methylation levels of KvDMR1. DNA methylation independent differences of gene expression were also found by others authors (Cordeiro et al., 2014; Piyasena et al., 2015; López-Abad et al., 2016). This result may reflect the intervention of trans-acting mechanisms that deregulate transcription, such as transcription factor binding. Even though our results were non-significant, we observed a tendency toward the increase of methylation in KvDMR1 CpGs, in IUGR cases. The CpG methylation is mainly catalyzed by differents DNA methyltransferases, thus the expression of *DNMT1* and *DNMT3A* was evaluated and compared between the groups. In the IUGR group was observed the overexpression of this genes, supporting the possibility of increased methylation. However, the expression of genes *DNMT1* and *DNMT3A* does not allow to draw conclusions concerning the methylation status of CpG KvDMR1.

The main limitation of this study was the small number of samples that were used to evaluate the methylation status. In future studies, the methylation analysis should be performed on a larger number of samples. However, since the technique used for evaluating methylation is time-consuming, an alternative procedure can be performed, such as MS-MLPA (Cordeiro et al., 2014), BS direct sequencing or Combined bisulfite restriction analysis (COBRA).

5-hydroxymethylation is a DNA modification that results from the 5mC oxidation by TET proteins. The alteration of this epigenetic modification was associated with cancer and neurodevelopment diseases (Zhu et al., 2015) and also with the size at birth (Piyasena et al., 2015). Reports demonstrated the 5hmC enrichment associated with overexpression of genes (Green et al., 2016). Others studies verified the differential distribution of the “sixth” base among several tissues, including the placenta (0,05-0,06% of 5hmC enrichment) (Li and Liu, 2011). The location of 5hmC in placenta was evaluated by Mora and his collaborators. They reported the 5hmC enrichment in CpG island shelves, that are regions with poor CpGs, and the 5hmC depletion in CpG island. Furthermore, the study demonstrated the 5hmC enrichment in gene body and in 5’ and 3’ UTR, in DMR of methylated allele and in the expressed allele of imprinted lncRNAs (observed in brain tissue). On the other hand, the 5hmC depletion was observed in the proximal promoter (Hernandez Mora et al., 2017).

In our study, the global 5hmC DNA was detected and quantified in all samples and compared between groups. The presence of 5hmC in placental samples with mean values of 0.073% (in cases control) and 0.05% (in cases IUGR) was similar to the percentage reported by Li and Liu, confirming the presence of this epigenetic

modification in placental tissue. The mean percentages of 5hmC in the two groups (IUGR vs control) were compared using t-test and was observed a tendency for 5hmC decrease in the IUGR samples ($p > 0.05$). Additionally, the evaluation of the expression of the genes encoding *TETs* was performed for a better understanding of the global hydroxymethylation of the samples. Of the three genes, *TET3* was the only gene with a significant difference between the study groups, being overexpressed in placental samples associated with IUGR. However, the lower expression of the *TET1*, observed in IUGR samples, supports the existence of a tendency for lower hydroxymethylation in this group. The investigation performed by Mora et al. evidenced an increased expression of *TET2* and *TET3* and a lower expression of *TET1* in the placental tissue than in the brain. Each TET protein may have specific function and may act in different genes regions. A report in human cancer cells demonstrated that the oxidation of 5-mC to 5-hmC occurs due to the TET1 enzyme and, on the other hand, TET2 and TET3 are responsible for the stimulation of the 5-hmC removal (Scourzic et al., 2015). In addition to this TET differential functions, other study report that the knockout of *Tet1* and *Tet2* was compensated by an increase in *Tet3* expression in mice (Rudenko et al., 2013). The same phenomenon may be related to our samples, since a negative correlation was observed between the expression of the *TET1* and *TET3* genes.

However, the absence of significant differences between groups in KvDMR1 methylation and global hydroxymetylation studies may reflect the action of other trans acting mechanisms. The lack of a significant difference in hydroxymethylation may also be due to the technique used, which only allows to analyze the global hydroxymethylation. Different regions may have different levels of hydroxymethylation (Green et al., 2016) and the global hydroxymethylation may differ from the levels of hydroxymetylation in imprinted gene (Piyasena et al., 2015). Therefore, a technique that allows a more specific hydroxymethylation analysis would be more advantageous for understanding the effect of hydroxymethylation on gene expression. In future studies, other technique should be used to infer the hydroxymethylated regions with a single-base resolution, such as oxBS-sequencing. This specific method allows the relative quantification and discrimination between 5mC and 5hmC in individual CpGs (Hernandez Mora et al., 2017).

VI. Conclusion

Intrauterine growth restriction is a serious pregnancy complication with high frequency and multifactoriality. For future treatment, it is necessary to study factors that can lead to IUGR and thus predict this disorder.

This study allowed to distinguish imprinted candidate genes that may be involved in the etiology of this disorder, such as *CDKN1C* and *PHLDA2*. The expression of these maternally expressed genes was significantly higher in term placentas IUGR, suggesting these two genes as potential IUGR biomarkers. In relation to *PEG10*, this gene also has an altered expression in placentas with the disorder, however, more studies will have to be established to understand if this derives from a consequence or a cause of IUGR. Thus, the evaluation of the *PEG10* expression in placentas IUGR at different trimester will be useful to understand the role of this gene in fetal growth.

The small sample size did not allow significant conclusions concerning the association of KvDMR1 methylation with gene expression and IUGR. The absence of significant differences in methylation between normal samples and IUGR samples may reflect the intervention of another trans-acting mechanism, leading to differential expression of some genes. Despite the non-significance of the results, in this study was observed a trend towards increased methylation of KvDMR1 in IUGR placentas. This assumption was also inferred with the observation of increased expression of the genes that code for enzymes responsible for the methylation process. Thus, more samples should be analyzed in later studies to validate this tendency.

This research was the first to evaluate the hydroxymethylation in placentas diagnosed with fetal growth restriction. The global presence of the epigenetic modification in the placental tissue was confirmed by the procedure used in this study. However, the difference between the two groups evaluated (IUGR vs control) was not significant. For that reason, the association between the hydroxymethylation, gene expression and the disorder cannot be established in this study. Further studies should evaluate this modification in specific genes related to growth retardation.

In conclusion, the results allowed the determination of two potential biomarkers for identification of pregnancies with risk of IUGR and confirmed the presence of the 5-hydroxymethylcytosine in placental tissue.

.

References

Albu, A.R., Horhoianu, I.A., Dumitrascu, M.C., Horhoianu, V., 2014. Growth assessment in diagnosis of Fetal Growth Restriction. Review. Journal of medicine and life 7, 150–4.

Allfrey, V.G., Faulkner, R., Mirsky, A.E., 1964. Acetylation and methylation of histones and their possible role in the regulation of rna synthesis. Proceedings of the National Academy of Sciences of the United States of America 51, 786–94.

Andrews, S.C., Wood, M.D., Tunster, S.J., Barton, S.C., Surani, A.M., John, R.M., 2007. Cdkn1c (p57Kip2) is the major regulator of embryonic growth within its imprinted domain on mouse distal chromosome 7. BMC Developmental Biology 7, 53. doi:10.1186/1471-213X-7-53

Apostolidou, S., Abu-Amero, S., O'Donoghue, K., Frost, J., Olafsdottir, O., Chavele, K.M., Whittaker, J.C., Loughna, P., Stanier, P., Moore, G.E., 2007. Elevated placental expression of the imprinted PHLDA2 gene is associated with low birth weight. Journal of Molecular Medicine 85, 379–387. doi:10.1007/s00109-006-0131-8

Arboleda, V.A., Lee, H., Parnaik, R., Fleming, A., Banerjee, A., Ferraz-de-Souza, B., Délot, E.C., Rodriguez-Fernandez, I.A., Braslavsky, D., Bergadá, I., Dell'Angelica, E.C., Nelson, S.F., Martinez-Agosto, J.A., Achermann, J.C., Vilain, E., 2012. Mutations in the PCNA-binding domain of CDKN1C cause IMAGe syndrome. Nature genetics 44, 788–92. doi:10.1038/ng.2275

Bamberg, C., Kalache, K.D., 2004. Prenatal diagnosis of fetal growth restriction. Seminars in Fetal and Neonatal Medicine 9, 387–394. doi:10.1016/j.siny.2004.03.007

Bannister, A.J., Kouzarides, T., 2011. Regulation of chromatin by histone modifications. Cell research 21, 381–95. doi:10.1038/cr.2011.22

Baschat, A., Galan, H., 2016. Intrauterine Gwoth Restriction, in: Obstetrics : Normal and Problem Pregnancies.

Beard, R.W., Nathanielsz, P.W., 2013. Fetal physiology and medicine : the basis of perinatology, 2nd ed. Butterworth-Heinemann.

Beatty, L., Weksberg, R., Sadowski, P.D., 2006. Detailed analysis of the methylation patterns of the KvDMR1 imprinting control region of human chromosome 11. Genomics 87, 46–56. doi:10.1016/j.ygeno.2005.05.015

Bergman, D., Halje, M., Nordin, M., Engström, W., 2013. Insulin-like growth factor 2 in development and disease: a mini-review. Gerontology 59, 240–9. doi:10.1159/000343995

Bianco-Miotto, T., Mayne, B.T., Buckberry, S., Breen, J., Rodriguez Lopez, C.M., Roberts, C.T., T, B.-M., Mayne B, B.S., J, B., Rodriguez Lopez C, R.C., 2016. Recent progress towards understanding the role of DNA methylation in human placental development. Reproduction (Cambridge, England) 152, R23-30. doi:10.1530/REP-16-0014

Bird, A., 2002. DNA methylation patterns and epigenetic memory. Genes & development 16, 6–21. doi:10.1101/gad.947102

Bock, C., Reither, S., Mikeska, T., Paulsen, M., Walter, J., Lengauer, T., 2005. BiQ Analyzer: visualization and quality control for DNA methylation data from bisulfite sequencing. Bioinformatics 21, 4067–4068. doi:10.1093/bioinformatics/bti652

Bourque, D.K.K., Avila, L., Peñaherrera, M., von Dadelszen, P., Robinson, W.P.P., 2010. Decreased Placental Methylation at the H19/IGF2 Imprinting Control Region is Associated with Normotensive Intrauterine Growth Restriction but not

Preeclampsia. Placenta 31, 197–202. doi:10.1016/j.placenta.2009.12.003

Bressan, F.F., De Bem, T.H.C., Perecin, F., Lopes, F.L.L., Ambrosio, C.E., Meirelles, F.V. V, Miglino, M.A.A., 2009. Unearthing the roles of imprinted genes in the placenta. Placenta 30, 823–34. doi:10.1016/j.placenta.2009.07.007

Calkins, K.L., Devaskar, S.U., 2015. Intrauterine Growth Restriction, Tenth Edit. ed, Fanaroff and Martin's Neonatal-Perinatal Medicine, 2-Volume Set. Elsevier Inc. doi:10.1016/B978-1-4557-5617-9.00016-6

Campos, D.A., Montenegro, N., Rodrigues, T., 2008. Protocolos de medicina materno-fetal, 2ª. ed. Lidel, Lisboa.

Chapman, C.G., Mariani, C.J., Wu, F., Meckel, K., Butun, F., Chuang, A., Madzo, J., Bissonette, M.B., Kwon, J.H., Godley, L.A., 2015. TET-catalyzed 5-hydroxymethylcytosine regulates gene expression in differentiating colonocytes and colon cancer. Scientific Reports 5, 17568. doi:10.1038/srep17568

Chen, H., Sun, M., Liu, J., Tong, C., Meng, T., 2015. Silencing of Paternally Expressed Gene 10 Inhibits Trophoblast Proliferation and Invasion. PloS one 10, e0144845. doi:10.1371/journal.pone.0144845

Chen, Z., Riggs, A.D., 2011. DNA methylation and demethylation in mammals. The Journal of biological chemistry 286, 18347–53. doi:10.1074/jbc.R110.205286

Chiesa, N., De Crescenzo, A., Mishra, K., Perone, L., Carella, M., Palumbo, O., Mussa, A., Sparago, A., Cerrato, F., Russo, S., Lapi, E., Cubellis, M.V., Kanduri, C., Cirillo Silengo, M., Riccio, A., Ferrero, G.B., 2012. The KCNQ1OT1 imprinting control region and non-coding RNA: new properties derived from the study of Beckwith-Wiedemann syndrome and Silver-Russell syndrome cases. Human molecular genetics 21, 10–25. doi:10.1093/hmg/ddr419

Constância, M., Hemberger, M., Hughes, J., Dean, W., Ferguson-Smith, A., Fundele, R., Stewart, F., Kelsey, G., Fowden, A., Sibley, C., Reik, W., 2002. Placental-specific IGF-II is a major modulator of placental and fetal growth. Nature 417, 945–948. doi:10.1038/nature00819

Cordeiro, A., Neto, A.P., Carvalho, F., Ramalho, C., Dória, S., 2014. Relevance of genomic imprinting in intrauterine human growth expression of CDKN1C, H19, IGF2, KCNQ1 and PHLDA2 imprinted genes. Journal of Assisted Reproduction and Genetics 31, 1361–1368. doi:10.1007/s10815-014-0278-0

De Crescenzo, A., Sparago, A., Cerrato, F., Palumbo, O., Carella, M., Miceli, M., Bronshtein, M., Riccio, A., Yaron, Y., 2013. Paternal deletion of the 11p15.5 centromeric-imprinting control region is associated with alteration of imprinted gene expression and recurrent severe intrauterine growth restriction. Journal of medical genetics 50, 99–103. doi:10.1136/jmedgenet-2012-101352

Demetriou, C., Abu-Amero, S., Thomas, A.C., Ishida, M., Aggarwal, R., Al-Olabi, L., Leon, L.J., Stafford, J.L., Syngelaki, A., Peebles, D., Nicolaides, K.H., Regan, L., Stanier, P., Moore, G.E., 2014. Paternally expressed, imprinted insulin-like growth factor-2 in chorionic villi correlates significantly with birth weight. PLoS ONE 9. doi:10.1371/journal.pone.0085454

Demicheva, E., Crispi, F., 2014. Long-term follow-up of intrauterine growth restriction: cardiovascular disorders. Fetal diagnosis and therapy 36, 143–53. doi:10.1159/000353633

Diaz-Meyer, N., 2003. Silencing of CDKN1C (p57KIP2) is associated with hypomethylation at KvDMR1 in Beckwith-Wiedemann syndrome. Journal of Medical Genetics 40, 797–801. doi:10.1136/jmg.40.11.797

Diplas, A.I., Lambertini, L., Lee, M.-J., Sperling, R., Lee, Y.L., Wetmur, J., Chen, J., 2009. Differential expression of imprinted genes in normal and IUGR human placentas. Epigenetics 4, 235–40.

Dória, S., Sousa, M., Fernandes, S., Ramalho, C., Brandão, O., Matias, A., Barros, A., Carvalho, F., 2010. Gene expression pattern of IGF2, PHLDA2, PEG10 and CDKN1C imprinted genes in spontaneous miscarriages or fetal deaths. Epigenetics 5, 444–50.

Dunger, D.B., Petry, C.J., Ong, K.K., 2006. Genetic Variations and Normal Fetal Growth. Hormone Research in Paediatrics 65, 34–40. doi:10.1159/000091504

Eggermann, T., Binder, G., Brioude, F., Maher, E.R., Lapunzina, P., Cubellis, M.V., Bergadá, I., Prawitt, D., Begemann, M., 2014. CDKN1C mutations: two sides of the same coin. Trends in molecular medicine 20, 614–22. doi:10.1016/j.molmed.2014.09.001

Figueras, F., Gardosi, J., 2011. Intrauterine growth restriction: New concepts in antenatal surveillance, diagnosis, and management. American Journal of Obstetrics and Gynecology. doi:10.1016/j.ajog.2010.08.055

Fowden, A.L., Forhead, A.J., 2015. Glucocorticoids as regulatory signals during intrauterine development. Experimental Physiology 100, 1477–1487. doi:10.1113/EP085212

Fowden, A.L., Forhead, A.J., 2009. Endocrine regulation of feto-placental growth. Hormone research 72, 257–65. doi:10.1159/000245927

Fowden, A.L., Forhead, A.J., Sferruzzi-Perri, A.N., Burton, G.J., Vaughan, O.R., 2015. Review: Endocrine regulation of placental phenotype. Placenta 36, S50–S59. doi:10.1016/j.placenta.2014.11.018

Fowden, A.L., Sibley, C., Reik, W., Constancia, M., 2006. Imprinted Genes, Placental Development and Fetal Growth. Hormone Research in Paediatrics 65, 50–58. doi:10.1159/000091506

Frost, J.M., Moore, G.E., 2010. The importance of imprinting in the human placenta. PLoS Genetics 6, 1–9. doi:10.1371/journal.pgen.1001015

Gaccioli, F., Lager, S., 2016. Placental Nutrient Transport and Intrauterine Growth Restriction. Frontiers in Physiology 7, 40. doi:10.3389/fphys.2016.00040

Gardosi, J., 2012. Normal Fetal Growth, in: Dewhurst's Textbook of Obstetrics & Gynaecology. Wiley-Blackwell, Oxford, UK, pp. 26–32. doi:10.1002/9781119979449.ch3

Gibney, E.R., Nolan, C.M., 2010. Epigenetics and gene expression. Heredity 105, 4–13. doi:10.1038/hdy.2010.54

Gicquel, C., Le Bouc, Y., 2006. Hormonal regulation of fetal growth. Hormone research 65 Suppl 3, 28–33. doi:10.1159/000091503

Green, B.B., Houseman, E.A., Johnson, K.C., Guerin, D.J., Armstrong, D.A., Christensen, B.C., Marsit, C.J., 2016. Hydroxymethylation is uniquely distributed within term placenta, and is associated with gene expression. FASEB Journal 30, 2874–2884. doi:10.1096/fj.201600310R

Greer, E.L., Shi, Y., 2012. Histone methylation: a dynamic mark in health, disease and inheritance. Nature reviews. Genetics 13, 343–57. doi:10.1038/nrg3173

Grimberg, A., Lifshitz, F., 2007. Worrisome Growth, in: Lifshitz, F. (Ed.), Pediatric Endocrinology - Volume 2. Informa Healthcare, New York, p. 816.

Gude, N.M., Roberts, C.T., Kalionis, B., King, R.G., 2004. Growth and function of the

normal human placenta. Thrombosis research 114, 397–407. doi:10.1016/j.thromres.2004.06.038

Guibert, S., Weber, M., 2013. Functions of DNA Methylation and Hydroxymethylation in Mammalian Development. pp. 47–83. doi:10.1016/B978-0-12-416027-9.00002-4

Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., Vandesompele, J., 2007. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. Genome biology 8, R19. doi:10.1186/gb-2007-8-2-r19

Hendrix, N., Berghella, V., 2008. Non-Placental Causes of Intrauterine Growth Restriction. Seminars in Perinatology 32, 161–165. doi:10.1053/j.semperi.2008.02.004

Hernandez Mora, J.R., Sanchez-Delgado, M., Petazzi, P., Moran, S., Esteller, M., Iglesias-Platas, I., Monk, D., 2017. Profiling of oxBS-450K 5-hydroxymethylcytosine in human placenta and brain reveals enrichment at imprinted loci. Epigenetics 00–00. doi:10.1080/15592294.2017.1344803

Hillman, S.L., Finer, S., Smart, M.C., Mathews, C., Lowe, R., Rakyan, V.K., Hitman, G.A., Williams, D.J., 2015. Novel DNA methylation profiles associated with key gene regulation and transcription pathways in blood and placenta of growth-restricted neonates. Epigenetics 10, 50–61. doi:10.4161/15592294.2014.989741

Hitchins, M.P., 2015. Constitutional epimutation as a mechanism for cancer causality and heritability? Nature Reviews Cancer 15, 625–634. doi:10.1038/nrc4001

Huntriss, J.D., Hemmings, K.E., Hinkins, M., Rutherford, A.J., Sturmey, R.G., Elder, K., Picton, H.M., 2013. Variable imprinting of the MEST gene in human preimplantation embryos. European Journal of Human Genetics 21, 40–47. doi:10.1038/ejhg.2012.102

Iglesias-Platas, I., Martin-Trujillo, A., Petazzi, P., Guillaumet-Adkins, A., Esteller, M., Monk, D., 2014. Altered expression of the imprinted transcription factor PLAGL1 deregulates a network of genes in the human IUGR placenta. Human molecular genetics 23, ddu347-. doi:10.1093/hmg/ddu347

Ishida, M., Moore, G.E., 2013. The role of imprinted genes in humans. Molecular Aspects of Medicine 34, 826–840. doi:10.1016/j.mam.2012.06.009

Janssen, A.B., Tunster, S.J., Heazell, A.E.P., John, R.M., 2016. Placental PHLDA2 expression is increased in cases of fetal growth restriction following reduced fetal movements. BMC medical genetics 17, 17. doi:10.1186/s12881-016-0279-1

Januar, V., Desoye, G., Novakovic, B., Cvitic, S., Saffery, R., 2015. Epigenetic regulation of human placental function and pregnancy outcome: Considerations for causal inference. American Journal of Obstetrics and Gynecology 213, S182–S196. doi:10.1016/j.ajog.2015.07.011

Jensen, A.B., Tunster, S.J., John, R.M., 2014. The significance of elevated placental PHLDA2 in human growth restricted pregnancies. Placenta 35, 528–532. doi:10.1016/j.placenta.2014.04.018

Kameswaran, V., Kaestner, K.H., 2014. The Missing lnc(RNA) between the pancreatic β-cell and diabetes. Frontiers in Genetics 5, 200. doi:10.3389/FGENE.2014.00200

Kanduri, C., 2016. Long noncoding RNAs: Lessons from genomic imprinting. Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms 1859, 102–111. doi:10.1016/j.bbagrm.2015.05.006

Katari, S., Turan, N., Bibikova, M., Erinle, O., Chalian, R., Foster, M., Gaughan, J.P., Coutifaris, C., Sapienza, C., 2009. DNA methylation and gene expression

differences in children conceived in vitro or in vivo. Human Molecular Genetics 18, 3769–3778. doi:10.1093/hmg/ddp319

Khoueiry, R., Ibala-Romdhane, S., Al-Khtib, M., Blachère, T., Lornage, J., Guérin, J.-. F., Lefèvre, A., 2013. Abnormal methylation of KCNQ1OT1 and differential methylation of H19 imprinting control regions in human ICSI embryos. Zygote 21, 129–138. doi:10.1017/S0967199411000694

Kinney, S.R.M., Pradhan, S., 2011. Regulation of Expression and Activity of DNA (Cytosine-5) Methyltransferases in Mammalian Cells, in: Progress in Molecular Biology and Translational Science. pp. 311–333. doi:10.1016/B978-0-12-387685-0.00009-3

Klug, M., Schmidhofer, S., Gebhard, C., Andreesen, R., Rehli, M., 2013. 5-Hydroxymethylcytosine is an essential intermediate of active DNA demethylation processes in primary human monocytes. Genome Biology 14, R46. doi:10.1186/gb-2013-14-5-r46

Ko, M., An, J., Bandukwala, H.S., Chavez, L., Äijö, T., Pastor, W.A., Segal, M.F., Li, H., Koh, K.P., Lähdesmäki, H., Hogan, P.G., Aravind, L., Rao, A., 2013. Modulation of TET2 expression and 5-methylcytosine oxidation by the CXXC domain protein IDAX. Nature 497, 122–126. doi:10.1038/nature12052

Kobayashi, S., Kohda, T., Miyoshi, N., Kuroiwa, Y., Aisaka, K., Tsutsumi, O., Kaneko-Ishino, T., Ishino, F., 1997. Human PEG1/MEST, an imprinted gene on chromosome 7. Human molecular genetics 6, 781–6.

Koppes, E., Himes, K.P., Chaillet, J.R., 2015. Partial Loss of Genomic Imprinting Reveals Important Roles for Kcnq1 and Peg10 Imprinted Domains in Placental Development. PloS one 10, e0135202. doi:10.1371/journal.pone.0135202

Koukoura, O., Sifakis, S., Soufla, G., Zaravinos, A., Apostolidou, S., Allison, J., Widschwendter, Martin Spandidos, D.A., 2011a. Loss of imprinting and aberrant methylation of IGF2 in placentas from pregnancies complicated with fetal growth restriction. International Journal of Molecular Medicine. doi:10.3892/ijmm.2011.754

Koukoura, O., Sifakis, S., Spandidos, D.A., 2012. DNA methylation in the human placenta and fetal growth (review). Molecular Medicine Reports 5, 883–889. doi:10.3892/mmr.2012.763

Koukoura, O., Sifakis, S., Zaravinos, A., Apostolidou, S., Jones, A., Hajiioannou, J., Widschwendter, M., Spandidos, D.A., 2011b. Hypomethylation along with increased H19 expression in placentas from pregnancies complicated with fetal growth restriction. Placenta 32, 51–7. doi:10.1016/j.placenta.2010.10.017

Krishna, U., Bhalerao, S., 2011. Placental insufficiency and fetal growth restriction. Journal of obstetrics and gynaecology of India 61, 505–11. doi:10.1007/s13224-011-0092-x

Kung, J.T.Y., Colognori, D., Lee, J.T., 2013. Long noncoding RNAs: past, present, and future. Genetics 193, 651–69. doi:10.1534/genetics.112.146704

Kurjak, A., Chervenak, F.A., 2006. Textbook of perinatal medicine. Informa Healthcare.

Lambertini, L., Lee, M.-J., Marsit, C.J., Chen, J., 2012. Genomic Imprinting in Human Placenta, in: Zheng, D.J. (Ed.), Recent Advances in Research on the Human Placenta. InTech, p. 428.

Lausman, A., McCarthy, F.P., Walker, M., Kingdom, J., 2012. OBSTETRICS Screening, Diagnosis, and Management of Intrauterine Growth Restriction. J Obstet Gynaecol Can 34, 17–28.

Lee, M.H., Reynisdóttir, I., Massagué, J., 1995. Cloning of p57KIP2, a cyclin-dependent

kinase inhibitor with unique domain structure and tissue distribution. Genes & development 9, 639–49.

Lee, S.-A., Ding, C., 2012. The dysfunctional placenta epigenome: causes and consequences. Epigenomics 4, 561–569. doi:10.2217/epi.12.49

Lennartsson, A., Ekwall, K., 2009. Histone modification patterns and epigenetic codes. Biochimica et Biophysica Acta (BBA) - General Subjects 1790, 863–868. doi:10.1016/j.bbagen.2008.12.006

Li, W., Liu, M., 2011. Distribution of 5-hydroxymethylcytosine in different human tissues. Journal of nucleic acids 2011, 870726. doi:10.4061/2011/870726

Li, Y., Tollefsbol, T.O., 2011. DNA Methylation Detection: Bisulfite Genomic Sequencing Analysis, in: Methods in Molecular Biology (Clifton, N.J.). pp. 11–21. doi:10.1007/978-1-61779-316-5_2

Lim, A.L., Ferguson-Smith, A.C., 2010. Genomic imprinting effects in a compromised in utero environment: Implications for a healthy pregnancy. Seminars in Cell & Developmental Biology 21, 201–208. doi:10.1016/j.semcdb.2009.10.008

López-Abad, M., Iglesias-Platas, I., Monk, D., 2016. Epigenetic Characterization of CDKN1C in Placenta Samples from Non-syndromic Intrauterine Growth Restriction. Frontiers in genetics 7, 62. doi:10.3389/fgene.2016.00062

Lyons, P., 2015. Intrauterine Growth Restriction, in: Obstetrics in Family Medicine. Springer International Publishing, Cham, pp. 45–53. doi:10.1007/978-3-319-20077-4_6

Maccani, M.A., Marsit, C.J., 2009. Epigenetics in the placenta. American journal of reproductive immunology (New York, N.Y. : 1989) 62, 78–89. doi:10.1111/j.1600-0897.2009.00716.x

Magalhães, H.R., Leite, S.B.P., Paz, C.C.P. de, Duarte, G., Ramos, E.S., 2013. Placental hydroxymethylation vs methylation at the imprinting control region 2 on chromosome 11p15.5. Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas 46, 916–919. doi:10.1590/1414-431X20133035

Maltepe, E., Fisher, S.J., 2015. Placenta: The Forgotten Organ. Annual Review of Cell and Developmental Biology 31, 523–552. doi:10.1146/annurev-cellbio-100814-125620

Marques, C.J., João Pinho, M., Carvalho, F., Bièche, I., Barros, A., Sousa, M., 2011. DNA methylation imprinting marks and DNA methyltransferase expression in human spermatogenic cell stages. Epigenetics 6, 1354–1361. doi:10.4161/epi.6.11.17993

Mayer, C., Joseph, K.S., 2013. Fetal growth: a review of terms, concepts and issues relevant to obstetrics. Ultrasound in Obstetrics & Gynecology 41, 136–145. doi:10.1002/uog.11204

McGrath, J., Solter, D., 1984. Completion of mouse embryogenesis requires both the maternal and paternal genomes. Cell 37, 179–83.

McMinn, J., Wei, M., Schupf, N., Cusmai, J., Johnson, E.B., Smith, A.C., Weksberg, R., Thaker, H.M., Tycko, B., 2006. Unbalanced Placental Expression of Imprinted Genes in Human Intrauterine Growth Restriction. Placenta 27, 540–549. doi:10.1016/j.placenta.2005.07.004

McNamara, G.I., Davis, B.A., Dwyer, D.M., John, R.M., Isles, A.R., 2016. Behavioural abnormalities in a novel mouse model for Silver Russell Syndrome. Human Molecular Genetics 33, ddw357. doi:10.1093/hmg/ddw357

Monk, D., 2015. Genomic imprinting in the human placenta. American journal of obstetrics and gynecology 213, S152-62. doi:10.1016/j.ajog.2015.06.032

Monk, D., Arnaud, P., Apostolidou, S., Hills, F.A., Kelsey, G., Stanier, P., Feil, R., Moore, G.E., 2006. Limited evolutionary conservation of imprinting in the human placenta. Proceedings of the National Academy of Sciences 103, 6623–6628. doi:10.1073/pnas.0511031103

Monk, D., Moore, G.E., 2004. Intrauterine growth restriction--genetic causes and consequences. Seminars in fetal & neonatal medicine 9, 371–378. doi:10.1016/j.siny.2004.03.002

Moore, G.E., Ishida, M., Demetriou, C., Al-Olabi, L., Leon, L.J., Thomas, A.C., Abu-Amero, S., Frost, J.M., Stafford, J.L., Chaoqun, Y., Duncan, A.J., Baigel, R., Brimioulle, M., Iglesias-Platas, I., Apostolidou, S., Aggarwal, R., Whittaker, J.C., Syngelaki, A., Nicolaides, K.H., Regan, L., Monk, D., Stanier, P., 2015. The role and interaction of imprinted genes in human fetal growth. Philosophical transactions of the Royal Society of London. Series B, Biological sciences 370. doi:10.1098/rstb.2014.0074

Moore, T., 1991. Genomic imprinting in mammalian development: a parental tug-of-war. Trends in Genetics 7, 45–49. doi:10.1016/0168-9525(91)90230-N

Morgan, H.D., Santos, F., Green, K., Dean, W., Reik, W., 2005. Epigenetic reprogramming in mammals. Human Molecular Genetics 14, R47–R58. doi:10.1093/hmg/ddi114

Mullis, P.-E., Tonella, P., 2008. Regulation of fetal growth: Consequences and impact of being born small. Best Practice & Research Clinical Endocrinology & Metabolism 22, 173–190. doi:10.1016/j.beem.2007.07.010

Murphy, V.E., Smith, R., Giles, W.B., Clifton, V.L., 2006. Endocrine Regulation of Human Fetal Growth: The Role of the Mother, Placenta, and Fetus. Endocrine Reviews 27, 141–169. doi:10.1210/er.2005-0011

Nelissen, E.C.M., van Montfoort, A.P.A., Dumoulin, J.C.M., Evers, J.L.H., 2011. Epigenetics and the placenta. Human Reproduction Update 17, 397–417. doi:10.1093/humupd/dmq052

Netchine, I., Rossignol, S., Azzi, S., Brioude, F., Le Bouc, Y., 2012. Imprinted anomalies in fetal and childhood growth disorders: the model of Russell-Silver and Beckwith-Wiedemann syndromes. Endocrine development 23, 60–70. doi:10.1159/000341750

Ono, R., Kobayashi, S., Wagatsuma, H., Aisaka, K., Kohda, T., Kaneko-Ishino, T., Ishino, F., 2001. A Retrotransposon-Derived Gene, PEG10, Is a Novel Imprinted Gene Located on Human Chromosome 7q21. Genomics 73, 232–237. doi:10.1006/geno.2001.6494

Ono, R., Nakamura, K., Inoue, K., Naruse, M., Usami, T., Wakisaka-Saito, N., Hino, T., Suzuki-Migishima, R., Ogonuki, N., Miki, H., Kohda, T., Ogura, A., Yokoyama, M., Kaneko-Ishino, T., Ishino, F., 2006. Deletion of Peg10, an imprinted gene acquired from a retrotransposon, causes early embryonic lethality. Nature Genetics 38, 101–106. doi:10.1038/ng1699

Oxvig, C., 2015. The role of PAPP-A in the IGF system: location, location, location. Journal of cell communication and signaling 9, 177–87. doi:10.1007/s12079-015-0259-9

Peleg, D., Kennedy, C.M., Hunter, S.K., 1998. Intrauterine growth restriction: identification and management. American family physician 58, 453–60, 466–7.

Peschansky, V.J., Wahlestedt, C., 2014. Non-coding RNAs as direct and indirect modulators of epigenetic regulation. Epigenetics 9, 3–12. doi:10.4161/epi.27473

Piedrahita, J.A., 2011. The role of imprinted genes in fetal growth abnormalities. Birth defects research. Part A, Clinical and molecular teratology 91, 682–92. doi:10.1002/bdra.20795

Piyasena, C., Reynolds, R.M., Khulan, B., Seckl, J.R., Menon, G., Drake, A.J., 2015. Placental 5-methylcytosine and 5-hydroxymethylcytosine patterns associate with size at birth. Epigenetics 10, 692–697. doi:10.1080/15592294.2015.1062963

Regnault, T.R.H., Limesand, S.W., Hay, Jr, W.W., 2001. Factors Influencing Fetal Growth. NeoReviews 2.

Resnik, R., Creasy, R.K., 2014. Chapter 47 - Intrauterine Growth Restriction, Seventh Ed. ed, Creasy and Resnik's Maternal-Fetal Medicine: Principles and Practice. Elsevier Inc. doi:10.1016/B978-1-4557-1137-6.00047-7

Rudenko, A., Dawlaty, M.M., Seo, J., Cheng, A.W., Meng, J., Le, T., Faull, K.F., Jaenisch, R., Tsai, L.-H., 2013. Tet1 Is Critical for Neuronal Activity-Regulated Gene Expression and Memory Extinction. Neuron 79, 1109–1122. doi:10.1016/j.neuron.2013.08.003

Salas, M., John, R., Saxena, A., Barton, S., Frank, D., Fitzpatrick, G., Higgins, M.J., Tycko, B., 2004. Placental growth retardation due to loss of imprinting of Phlda2. Mechanisms of Development 121, 1199–1210. doi:10.1016/j.mod.2004.05.017

Saleem, T., Sajjad, N., Fatima, S., Habib, N., Ali, S.R., Qadir, M., 2011. Intrauterine growth retardation - small events, big consequences. Italian Journal of Pediatrics 37, 41. doi:10.1186/1824-7288-37-41

Sankaran, S., Kyle, P.M., 2009. Aetiology and Pathogenesis of IUGR. Best Practice & Research Clinical Obstetrics & Gynaecology 23, 765–777. doi:10.1016/j.bpobgyn.2009.05.003

Schreiner, F., Gohlke, B., Stutte, S., Bartmann, P., Hecher, K., Oldenburg, J., El-Maarri, O., Woelfle, J., 2014. 11p15 DNA-methylation analysis in monozygotic twins with discordant intrauterine development due to severe twin-to-twin transfusion syndrome. Clinical epigenetics 6, 6. doi:10.1186/1868-7083-6-6

Schroeder, D.I., LaSalle, J.M., 2013. How has the study of the human placenta aided our understanding of partially methylated genes? Epigenomics 5, 645–654. doi:10.2217/epi.13.62

Scifres, C.M., Nelson, D.M., 2009. Intrauterine growth restriction, human placental development and trophoblast cell death. The Journal of Physiology 587, 3453–3458. doi:10.1113/jphysiol.2009.173252

Scourzic, L., Mouly, E., Bernard, O.A., 2015. TET proteins and the control of cytosine demethylation in cancer. Genome medicine 7, 9. doi:10.1186/s13073-015-0134-6

Sharma, D., Sharma, P., Shastri, S., 2017. Genetic, metabolic and endocrine aspect of intrauterine growth restriction: an update. The Journal of Maternal-Fetal & Neonatal Medicine 30, 1–13. doi:10.1080/14767058.2016.1245285

Sharma, D., Shastri, S., Farahbakhsh, N., Sharma, P., 2016a. Intrauterine growth restriction ? part 1. The Journal of Maternal-Fetal & Neonatal Medicine 29, 3977–3987. doi:10.3109/14767058.2016.1152249

Sharma, D., Shastri, S., Sharma, P., 2016b. Intrauterine Growth Restriction: Antenatal and Postnatal Aspects. Clinical medicine insights. Pediatrics 10, 67–83. doi:10.4137/CMPed.S40070

Shi, X., He, Z., Gao, Y., Luo, Y., Gou, C., Fang, Q., 2014. Placental expression of PHLDA2 in selective intrauterine growth restriction in monozygotic twins. Placenta 35, 428–430. doi:10.1016/j.placenta.2014.03.006

Shmela, M.E., Gicquel, C.F., 2013. Human diseases versus mouse models: insights into the regulation of genomic imprinting at the human 11p15/mouse distal chromosome 7 region. Journal of Medical Genetics 50, 11–20. doi:10.1136/jmedgenet-2012-101321

Sibley, C.P., Coan, P.M., Ferguson-Smith, A.C., Dean, W., Hughes, J., Smith, P., Reik, W., Burton, G.J., Fowden, A.L., Constancia, M., 2004. Placental-specific insulin-like growth factor 2 (Igf2) regulates the diffusional exchange characteristics of the mouse placenta. Proceedings of the National Academy of Sciences 101, 8204–8208. doi:10.1073/pnas.0402508101

Singh, V.B., Sribenja, S., Wilson, K.E., Attwood, K.M., Hillman, J.C., Pathak, S., Higgins, M.J., 2017. Blocked transcription through KvDMR1 results in absence of methylation and gene silencing resembling Beckwith-Wiedemann syndrome. Development 144, 1820–1830. doi:10.1242/dev.145136

Smith, G.C.S., Lees, C.C., 2012. Disorders of Fetal Growth and Assessment of Fetal Well-Being, in: Dewhurst's Textbook of Obstetrics & Gynaecology. Wiley-Blackwell, Oxford, UK, pp. 200–207. doi:10.1002/9781119979449.ch18

Sood, R., Zehnder, J.L., Druzin, M.L., Brown, P.O., 2006. Gene expression patterns in human placenta. Proceedings of the National Academy of Sciences of the United States of America 103, 5478–83. doi:10.1073/pnas.0508035103

Stolp, H., Neuhaus, A., Sundramoorthi, R., Molnár, Z., 2012. The Long and the Short of it: Gene and Environment Interactions During Early Cortical Development and Consequences for Long-Term Neurological Disease. Frontiers in Psychiatry 3. doi:10.3389/fpsyt.2012.00050

Suhag, A., Berghella, V., 2013. Intrauterine Growth Restriction (IUGR): Etiology and Diagnosis. Current Obstetrics and Gynecology Reports 2, 102–111. doi:10.1007/s13669-013-0041-z

Surani, M.A.H., Barton, S.C., Norris, M.L., 1984. Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis. Nature 308, 548–550. doi:10.1038/308548a0

Tunster, S.J., Creeth, H.D.J., John, R.M., 2016. The imprinted Phlda2 gene modulates a major endocrine compartment of the placenta to regulate placental demands for maternal resources. Developmental Biology 409, 251–260. doi:10.1016/j.ydbio.2015.10.015

Tunster, S.J., Tycko, B., John, R.M., 2010. The Imprinted Phlda2 Gene Regulates Extraembryonic Energy Stores. Molecular and Cellular Biology 30, 295–306. doi:10.1128/MCB.00662-09

Tunster, S.J., Van de Pette, M., John, R.M., 2011. Fetal overgrowth in the Cdkn1c mouse model of Beckwith-Wiedemann syndrome. Disease models & mechanisms 4, 814–21. doi:10.1242/dmm.007328

Tunster, S.J., Van De Pette, M., John, R.M., 2014. Isolating the role of elevated Phlda2 in asymmetric late fetal growth restriction in mice. Disease Models & Mechanisms 7.

Uauy, R., Casanello, P., Krause, B., Kuzanovic, J.P., Corvalan, C., 2013. Conceptual basis for prescriptive growth standards from conception to early childhood: Present and future. BJOG: An International Journal of Obstetrics and Gynaecology 120, 3–8. doi:10.1111/1471-0528.12057

Unek, G., Ozmen, A., Ozekinci, M., Sakinci, M., Korgun, E.T., 2014. Immunolocalization of cell cycle proteins (p57, p27, cyclin D3, PCNA and Ki67) in intrauterine growth retardation (IUGR) and normal human term placentas. Acta Histochemica 116, 493–502. doi:10.1016/j.acthis.2013.10.007

Vaissière, T., Sawan, C., Herceg, Z., 2008. Epigenetic interplay between histone modifications and DNA methylation in gene silencing. Mutation Research/Reviews in Mutation Research 659, 40–48. doi:10.1016/j.mrrev.2008.02.004

Wilkins-Haug, L., Quade, B., Morton, C.C., 2006. Confined placental mosaicism as a risk factor among newborns with fetal growth restriction. Prenatal Diagnosis 26, 428–432. doi:10.1002/pd.1430

Williams, R.L., Creasy, R.K., Cunningham, G.C., Hawes, W.E., Norris, F.D., Tashiro, M., 1982. Fetal growth and perinatal viability in California. Obstetrics and gynecology 59, 624–32.

Yuen, R.K.C., Avila, L., Peñaherrera, M.S., von Dadelszen, P., Lefebvre, L., Kobor, M.S., Robinson, W.P., 2009. Human Placental-Specific Epipolymorphism and its Association with Adverse Pregnancy Outcomes. PLoS ONE 4, e7389. doi:10.1371/journal.pone.0007389

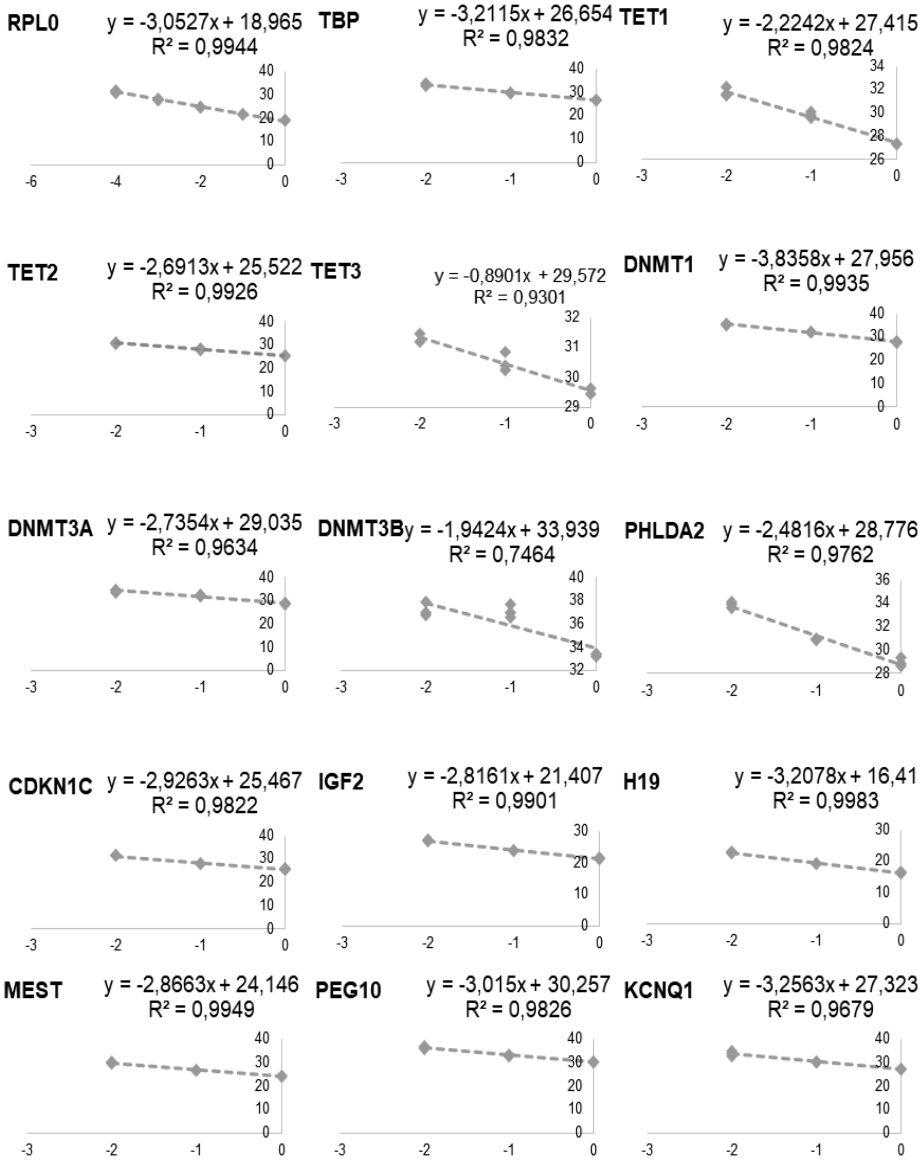
Zhang, J., Merialdi, M., Platt, L.D., Kramer, M.S., 2010. Defining normal and abnormal fetal growth: promises and challenges. American journal of obstetrics and gynecology 202, 522–8. doi:10.1016/j.ajog.2009.10.889

Zhu, H., Wang, G., Qian, J., 2016. Transcription factors as readers and effectors of DNA methylation. Nature Reviews Genetics 17, 551–565. doi:10.1038/nrg.2016.83

Zhu, L., Lv, R., Kong, L., Cheng, H., Lan, F., Li, X., 2015. Genome-wide mapping of 5mC and 5hmC identified differentially modified genomic regions in late-onset severe preeclampsia: A pilot study. PLoS ONE 10, 1–15. doi:10.1371/journal.pone.0134119

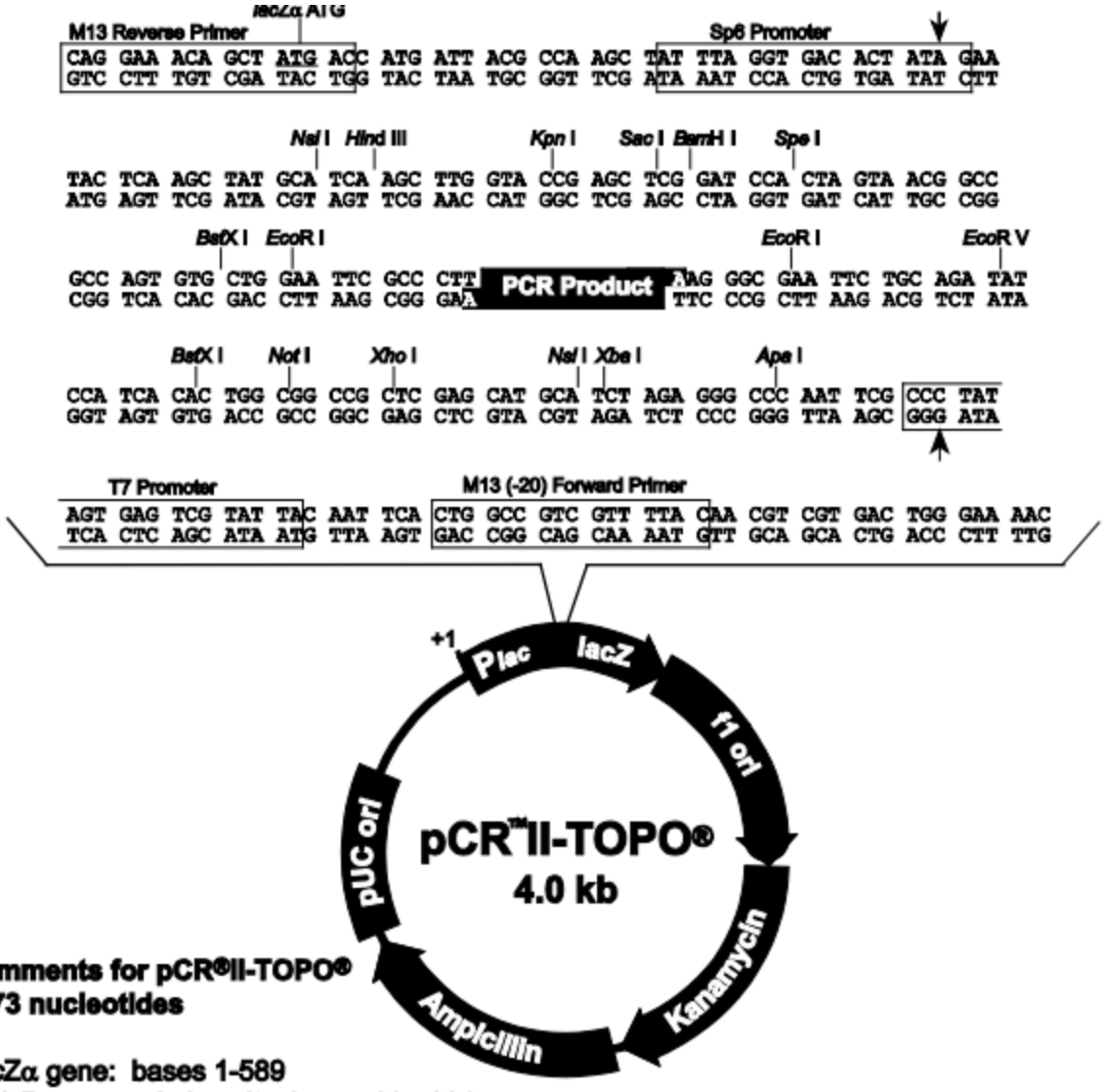
Appendix

qRT-PCR Standard curves for all genes



Gene	Amplification efficiency (%)
TBP	105
RPL0	113
TET1	182
TET2	135
TET3	1228
DNMT1	82
DNMT3A	132
DNMT3B	227
PHLDA2	153
CDKN1C	120
IGF2	127
MEST	124
PEG10	115
H19	105
KCNQ1	103

pCR™II-TOPO® map



Comments for pCR®II-TOPO®
3973 nucleotides

LacZα gene: bases 1-589
M13 Reverse priming site: bases 205-221
Sp6 promoter: bases 239-256
Multiple Cloning Site: bases 269-383
T7 promoter: bases 406-425
M13 (-20) Forward priming site: bases 433-448
f1 origin: bases 590-1027
Kanamycin resistance ORF: bases 1361-2155
Ampicillin resistance ORF: bases 2173-3033
pUC origin: bases 3178-3851