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Amilcar José Garcia Cordeiro

# RELEVANCE OF GENOMIC IMPRINTING IN INTRAUTERINE HUMAN GROWTH – Expression of imprinted genes IGF2, PHLDA2, CDKN1C and KCNQ1 in fetal growth restriction

**março, 2014**

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**Área: Genética Médica**

**Trabalho efetuado sob a Orientação de:**

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**E sob a Coorientação de:**

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Relevance of genomic imprinting in intrauterine human growth - Expression of imprinted genes IGF2, PHLDA2, CDKN1C and KCNQ1 in fetal growth restriction

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RELEVANCE OF GENOMIC IMPRINTING IN INTRAUTERINE HUMAN GROWTH
Expression of imprinted genes *IGF2*, *PHLDA2*, *CDKN1C* and *KCNQ1* in fetal growth restriction.

**Abstract**

*Purpose:* Relation of imprinted genes (*IGF2, CDKN1C, PHLDA2 and KCNQ1*) with human fetal growth.

*Methods:* RNA was extracted from fetuses with growth restriction and from the control group without growth restriction. cDNA synthesis was performed to evaluate the gene expressions patterns of *IGF2, CDKN1C, PHLDA2, H19* and *KCNQ1* genes using RT-PCR. The housekeeping gene *GAPDH* was used as control. DNA extraction and MS-MLPA were also performed to assess the IC1 and IC2 DNA methylation status on chromosome 11p15.5.

*Results:* The expression levels of IGF2 in the growth restriction group were statistically down-regulated ($p < 0.0001$). Sixty five percent of the cases with IUGR were hypomethylated in IC1.

*Conclusions:* The genomic imprinting is a phenomenon that plays a very important role in the fetal and placental development. Several imprinting genes may regulate this process and despite the several studies performed before additional research is still required. This study allows us to stress the importance of IGF2 gene during the pregnancy. Hypomethylation of the IC1 on chromosome 15p15.5 and down-regulation of the *IGF2* gene seems to be associated with intrauterine growth restriction.

**Keywords:** Imprinted genes, RT-PCR, DNA Methylation, IUGR

## Introduction

Intrauterine growth restriction (IUGR) is a condition in which a fetus is unable to achieve its genetically determined potential size thereby increasing their perinatal risk of morbidity and mortality. IUGR encompasses many different maternal and fetal causes. Concerning fetal problems, chromosomal abnormalities are one of the major causes of growth restriction [1]. Additionally, this phenomenon may happen due to an abnormal gene expression in the tissues leading to an abnormal growth of the fetus.

Epigenetics has a central role in the regulation of fetal growth and development. In particular, imprinted genes comprise a small subset of the human genome that has been shown to be essential to fetal, placental and behavioral development. Genomic imprinting is characterized by an epigenetic modification in which one allele is repressed according to parental origin. In general, paternally expressed genes promote fetal and placental growth while maternally expressed genes limits conceptus growth. This is consistent with the imprinting conflict theory, which postulates that the paternal genome maximizes extraction of maternal resources for the benefit of the paternal offspring while maternal genome limits nutrient provision acting to preserve and distribute such resources more equally between all her potential offspring [2-4]. Currently, there are over 90 known human imprinted genes in man (http://www.geneimprint.com). Insulin-like growth factor 2 (*IGF2*) is a paternally expressed gene, which acts as growth factor and has been associated with some pathologies such as Beckwith-Wiedmann syndrome (BWS, OMIM: #130650), which is characterized among other by an overgrowth [5, 6]. Having the opposite effect, Cyclin-dependent kinase inhibitor 1C (*CDKN1C*) [7], Pleckstrin homology-like domain family A member 2 (*PHLDA2*) and Potassium voltage-gated channel (*KCNQ1*) are imprinted genes that tend to restrict fetal body's weight, being associated with restriction growth syndromes such as Silver-Russell syndrome (SRS, OMIM: #180860) [8, 9]. All these genes are located in the same cluster, on chromosome 11 at p15.5. Two independent imprinting control regions or imprinting centers (IC), IC1 and IC2, regulate its expression. *IGF2* and *H19* imprinting genes are co-localized in the IC1, which contains a methylation-sensitive chromatin insulator that is responsible for controlling the access to them. Therefore, methylation in the IC1 of the paternal chromosome prevents the biding of the transcription factor CTCF, being *H19* hypermethylated and silenced and allowing the expression of *IGF2*. On the other hand, in the maternal chromosome, the lack of methylation in this region prevents the activation of *IGF2* and the presence of the insulator CTCF facilitates *H19* transcription.

In humans, deregulation of the *IGF2/H19* imprinted region is associated with the overgrowth and tumor predisposition-related BWS and with SRS, mainly characterized by pre- and post-natal growth deficiency.

IC2 is much larger and responsible for controlling several maternally expressed genes namely *CDKN1C, KCNQ1* and *PHLDA2*. It also includes a non-coding *KCNQ1OT1* gene that has an antisense orientation with respect to the protein-coding gene *KCNQ1*. In the maternal chromosome, IC2 is methylated, *KCNQ1OT1* is not transcribed and the flanking imprinted genes are expressed. On the paternal chromosome the IC2 sequence itself and/or the *KCNQ1OT1* transcript mediate the silence of the several genes of the region including the growth inhibitors *CDKN1C, PHLDA2* and *KCNQ1* genes (Fig 2A) [10-12].

Genomic DNA methylation in these ICs may exhibit substantial variation among human individuals. This epigenetic variation might contribute either to phenotypic variation, as well as to human disease [12, 13]. Although this subject has been the focus of much attention recently, the role of human imprinted genes in fetal development is not fully understood. With this study we assess the expression levels of four imprinted genes (*IGF2, CDKN1C, PHLDA2* and *KCNQ1*) in fetal and placental samples from spontaneous abortions or fetal deaths in which has been identified growth restriction. Additionally, we studied the IC1 and IC2 methylation status on chromosome 11p15.5 in the same samples.

## Materials and Methods

### Tissue Samples

We collected a total of 25 tissue samples from 13 fetuses; 10 of them were diagnosed with growth restriction and 3 of them without restriction, forming the control group. The samples were obtained from fetal tissue (n=12) and placental tissue (n=13). In the growth restriction group 9/10 cases have paired fetal/placental samples, while in the control group this is seen in 2/3 cases. The cases resulted from spontaneous miscarriage or fetal death in which has been identified growth restriction. The controls were selected from those with an infectious cause identified.

All samples were from fetus in the second trimester, with a gestational age between 14 and 24 weeks.

The samples were collected in RNA later and stored at -80ºC until use. Only cases with normal karyotype were used in our study.

The experimental plan was approved by the ethical committee of Centro Hospitalar de São João, EPE.

**RNA extraction and cDNA synthesis**

Total RNA was extracted from samples using 1ml of Tripure Isolation Reagent (Roche, Diagnostics, Indianapolis, IN, USA) according to the manufacturer's standard protocol. Quantification and purity were determined by NanoDrop 2000 UV-Vis Spectrophotometer (Nanodrop Technologies, Wilmington, USA). For cDNA synthesis, 10µg of total RNA was subjected to reverse transcription using qScript™ cDNA Synthesis Kit (Quanta BioSciences, Inc., Gaithersburg, USA) following the manufacturer's instructions.

**Quantitative RT-PCR**

RNA expression levels of four imprinted genes (*CDKN1C, IGF2, KCNQ1* and *PHLDA2*) and one housekeeping gene (*GADPH*) were analyzed by Real-Time PCR on a StepOnePlus™ Real-Time PCR System (Life Technologies Corporation, California, USA). Taqman® Gene Expression Assays (Life Technologies Corporation, California, USA) were used for each target gene *CDKN1C* (Hs00175938_m1), *IGF2* (Hs01005963_m1), *KCNQ1* (Hs00923522_m1) and *PHLDA2* (Hs00169368_m1), and endogenous control *GAPDH* (Hs99999905_m1). PCR reactions were performed in a 25 µl volume containing 5 µl of cDNA, 12.5 µl of TaqMan® Universal PCR Master Mix System (Life Technologies Corporation, California, USA), 6.25 µl of Rnase-free water and 1.25 µl of 20X TaqMan ® Gene Expression Assay Mix for each gene. PCR was performed in separated wells for each reaction and each sample was run in triplicate. For each gene, cases and controls samples were run in the same RT-PCR plate to minimize intra-plate variations. PCR parameters were as follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. In each plate negative controls were included.

**DNA extraction and MS-MLPA**

DNA extraction was performed according to the manufacturer's standard protocol using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany).

Afterward, MS-MLPA was performed on genomic DNA with SALSA MS-MLPA kit ME030-C1 BWS/SRS, (MRC-Holland, Amsterdam, The Nederland's), according to the manufacturer's instructions. This mix contains 42 probes, 26 of which for the region 11p.15. The methylation status of this region can also be determined by 10 of these probes since they contain an *HhaI* recognition site. In addition, further 15 probes are added for reference.

After 16 h of hybridization at 60ºC, samples were equally split into two aliquots. The first aliquot underwent ligation only, whereas the second one underwent ligation followed by enzymatic digestion with *HhaI* (a restriction enzyme recognizing only unmethylated DNA) and PCR amplification using universal primers. In the latter case, amplification products were obtained and detected by capillary electrophoresis only if the CpG included in the *HhaI* site was methylated and, therefore, not digested. Ligation, enzymatic digestion, and PCR amplification were performed according to the manufacturer's instructions. PCR products (1 ml) were mixed with 0.3 ml of internal size standard (GeneScan™ 600 LIZ; Applied Biosystems) and 13,7 ml of deionized formamide, and injected into 3500 Genetic Analyzer (Life Technologies Corporation, California, USA).

**Data Analysis**

*Gene Expression Values*

The results were treated using the validated Livak method [14].

A mean was calculated between the 3 replicates for the reference gene and the target gene. The value of threshold cycle (CT) of the target gene was deducted to that of reference (ref) or housekeeping gene, for the test samples [ΔCT (sample) = CT (ref) – CT (target gene)]. Later, the expression ratio was considered by calculating the normalized expression ($2^{-\Delta CT}$ = normalized expression ratio)

The result obtained of the target gene in the sample was therefore normalized to the expression of a reference gene. Normalizing the expression of the target gene to that of the reference gene compensates for any difference in the amount of the sample cDNA.

*MS-MLPA*

Each probe's signal was divided by the signal of each reference probe in the sample. After, the median of these ratios was estimated, so called Normalization Constant (NC). This value was divided by the average NC obtained in the undigested reference samples. Values ranging from 0.65 to 1.35 were considered as having normal number copies (diploid); a deletion was suspected for ratio less than 0.65 and a duplication was suspected for ratio more than 1.35. Quantification of the methylation status of a CpG site was done by dividing the NC of each MS-MLPA probe obtained on the digested aliquot by the NC of each MS-MLPA probe obtained on the corresponding undigested aliquot. To simplify the interpretation of data, we calculated the average methylation status obtained by the *H19* and *KCNQ1OT1* probes and indicated them as IC1 and IC2 methylation index, respectively. Due to our reduced numbers of controls, previous published data was used for the determination of the normal methylation levels ranges, so the mean methylation indices for normal samples used were 0.52 (range 0.47-0.58) for IC2 and 0.50 (range 0.46-0.55) for IC1 [11].

## Results

**RT-PCR Expression Profile**

A total of 20 cDNA samples derived from fetuses diagnosed with growth restriction and 5 cDNA samples derived from healthy fetuses (control group) were analyzed by RT-PCR for gene expression quantification (Fig. 1). The results indicate that the expression levels of *IGF2* in the growth restriction group were statistically down-regulated ($p < 0.0001$). The expression profiles for the other studied genes showed no statistical difference.

**DNA methylation analysis**

A total of 25 DNA samples derived from fetuses diagnosed with growth restriction (n=20) and from healthy fetuses, control group (n=5), were analyzed to evaluate the methylation status. The results are summarized in Table 1. No copy number alterations were found.

Four out of 20 samples with growth restriction presented a normal methylation status in the IC1, 13 out of 20 had IC1 hypomethylation and 3 out of 20 had IC1 hypermethylated. In the control group one sample out of 5 presented a normal methylation status in the IC1, and 4 out of 5 had IC1 hypermethylated.

Regarding IC2 in the growth restriction group, a normal methylation status was detected in 11 out of 20 samples, 8 out of 20 had IC2 hypomethylation and 1 out of 20 had IC2 hypermethylation. In the control group 1 out of 5 had a normal methylation status and the other 4 out of 5 had IC2 hypomethylation.

## Discussion

IUGR comprises one of the leading obstetric complications, indicating the presence of a pathophysiologic process occurring in utero that inhibits fetal growth. This regulation involves multiple factors with complex mechanisms. In order to better elucidate this process we studied four imprinted genes, *CDKN1C, IGF2, KCNQ1* and *PHLDA2,* since they play an important role in this area. Therefore, mRNA expression of these imprinted genes in IUGR and controls cases was analyzed.

The results from the mRNA expression studies indicated that *IGF2* gene levels were downregulated in the IUGR group. The expression profiles of the other genes showed no statistical difference. Regarding *IGF2*, the majority of reports have focused on evaluation of the expression level change in the fetal/placental unit, in association with intrauterine growth defects. In spite of some conflicting evidence regarding downregulation levels in the placenta of IUGR, some authors describe decreased expression in association with intrauterine growth restriction [15-18]. Sibley *et al*., reported downregulation of *IGF2* in cases with IUGR placenta versus normal term placenta, albeit with greater case-to-case variability [16]. In mouse, mutations in *igf2* gene lead to IUGR [19].

*IGF2* is considered the major fetal growth factor that regulates fetal/placental growth by stimulating trophoblastic migration and invasion. It also regulates diffusional exchange characteristics of the placenta. Although, intrauterine growth restriction is an extremely complex phenomenon, other genes and processes could contribute for this particular condition [2].

In this study, we did not find statistical differences between the two groups referred above in respect to the other three genes included in the study. Nevertheless, in a previous study done by our group, we observed an increase in the *PHLDA2* expression levels gene associated with spontaneous abortions, in the first trimester, and with IUGR in second trimester cases, although not very significant [20]. It is important to stress that it has also been suggested that *PHLDA2* gene may have a more profound effect on the placenta at early gestational ages when the placenta is more active, which is also in accordance with our previous report [18, 20].

Additionally to the mRNA expression levels, the study of the IC1 and IC2 methylation status was carried out in order to evaluate if abnormalities in these imprinting centres could be related with the IUGR. MS-MLPA was used for this study because it was already demonstrated that is a very specific and sensitive method, allowing the detection of both copy number and methylation level of the investigated loci [11].

*H19* and *IGF2* share common enhancers located downstream of *H19*. IC1 prevents *IGF2* expression from the maternal allele but allows its expression from the paternal allele, due to the DNA methylation mark at IC1. This epigenetic mark is extended to the *H19* promoter on the paternal allele, restricting *H19* expression to the maternal chromosome. Sixty percent of SRS cases are associated with loss of DNA methylation (LOM) from the paternal IC1 allele [8]. This defect represents a maternalization of IC1; this means that the methylated paternal allele acquires a maternal epigenotype (Fig. 2A and B). Consequently, it is expected a decrease of *IGF2* expression and/or an increase in H19 expression and this may be associated with growth restriction, which is one of the main characteristics of the SRS phenotype. The same molecular defect can be applied to IUGR cases.

Our results showed that 65% of the cases with IUGR were hypomethylated in IC1. This means that IC1 hypomethylation could be one of the possible explanations for the fetal growth restriction.

Several studies revealed IC1/IC2 epimutations in patients with SRS. In one study MS-MLPA performed in peripheral blood from three patients referred as SRS revealed hypomethylation in both IC1 and IC2 but when the

study was performed using buccal cells only the hypomethylation of the IC1 was confirmed [21]. This means that different tissues can have different methylations ranges. In present study we used fetal and placental tissues from IUGR cases, however due to small number of cases it was not possible to compare these different tissues. It will be very important to enlarge the series of IUGR cases, assessing different tissues (fetal and placental) and also extending the study to the third trimester of pregnancy.

In the present study, hypomethylation of IC2 was also observed for 40% of the samples. Begemann *et al.* and Azzi *et al.* found 3.8% and 4.05% of SRS cases with IC2 hypomethylation, in previous studies, respectively [21, 22]. The specific reason for the development of IUGR is not clear, nevertheless mosaicism could explain variability, and the loss of paternal or maternal methylation marks could probably not only be attributed to a deficient acquisition of methylation during gametogenesis but be consistent with an incorrect maintenance of methylation after fertilization. Spontaneous abortions cases could reflect more severe cases with extreme methylation variations.

In conclusion, several factors can affect fetal growth as maternal nutritional status, diet and exposure to environmental factors. This leads to an alteration in nutrient availability to the fetus and to a modulation of placental gene expression [15]. Imprinted genes can be responsible for IUGR, but more other non-imprinted genes may be involved [2].

It would be interesting to compare our data from the second trimester with data from the third trimester and assess whether there are differences throughout gestation.

More research is needed in this field, because there is not much knowledge about imprinting gene regulation, as well as their proper role in placental and human fetal growth. The possibility to early identification of differences in the methylation ranges (i.e. hypomethylation of IC1 using MS-MLPA) could be used to identify pregnancies with higher risk of severe IUGR.

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Fig. 1: Expression levels of the studied genes. cDNA expression was normalized using a housekeeping gene (*GAPDH*). Error bars represents standard error of the mean (SEM). Significant differences between groups are represented as: * $p < 0.0001$. The data was analysed by Student's t-test. GR – Growth Restriction.

Fig. 2: Regulation of imprinted genes expression according to IC1/IC2 methylation, both in paternal (pat) and maternal (mat) chromosomes; (A) – Epigenotypes of normal imprinted gene expression, (B) – Epigenetic abnormalities often detected in SRS patients, representing maternalization of IC1.

Table 1: Summary of MS-MLPA methylation analysis.

| | Methylation Status | IC1<br>Nº Samples<br>($M \pm SD$) | IC2<br>Nº Samples<br>($M \pm SD$) |
|---|---|---|---|
| Growth Restriction | Normal | 4 (51.9 ± 1.7) | 11 (51.4 ± 3.3) |
| | Hypomethylated | 13 (37.5 ± 4.5) | 8 (40.4 ± 7.7) |
| | Hypermethylated | 3 (60.0 ± 5.7) | 1 (58.2) |
| Controls | Normal | 1 (51.6) | 1 (56.3) |
| | Hypomethylated | 0 (0) | 0 (0) |
| | Hypermethylated | 4 (56.0 ± 0.4) | 4 (62.6 ± 4.6) |

Fig. 1

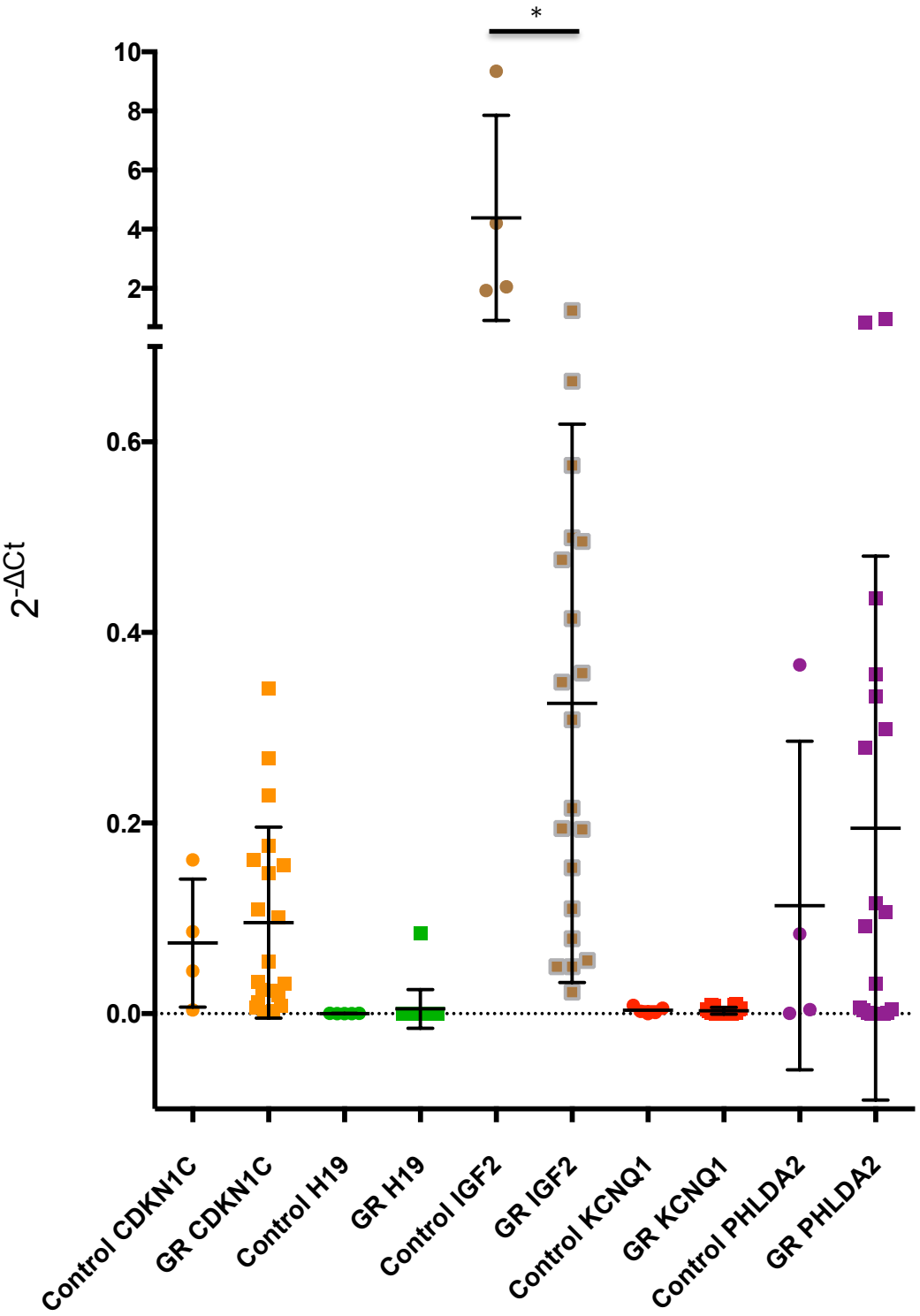
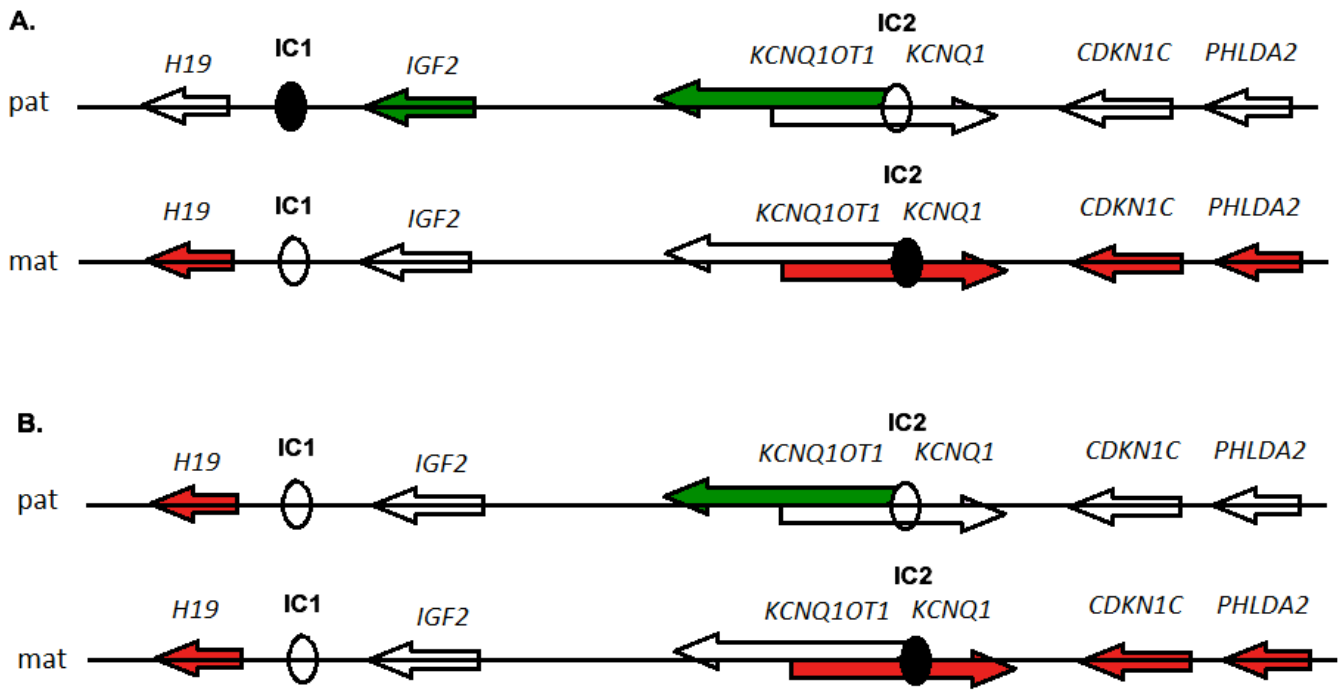

*
10
8
6
4
2
0.6
0.4
0.2
0.0
2-ΔCt
Control CDKN1C
GR CDKN1C
Control H19
GR H19
Control IGF2
GR IGF2
Control KCNQ1
GR KCNQ1
Control PHLDA2
GR PHLDA2

Fig. 2


A.
pat
H19
IC1
IGF2
IC2
KCNQ1OT1
KCNQ1
CDKN1C
PHLDA2
mat
H19
IC1
IGF2
IC2
KCNQ1OT1
KCNQ1
CDKN1C
PHLDA2
B.
pat
H19
IC1
IGF2
IC2
KCNQ1OT1
KCNQ1
CDKN1C
PHLDA2
mat
H19
IC1
IGF2
IC2
KCNQ1OT1
KCNQ1
CDKN1C
PHLDA2

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À Doutora Carla Ramalho agradeço o entusiasmo, disponibilidade, não podendo deixar de agradecer pela visão crítica sobre o trabalho.

À Doutora Filipa Carvalho agradeço o apoio prestado e a fomentação da discussão dos resultados.

À Mestre Ana Paula Neto agradeço o apoio prestado, a ajuda constante no laboratório e a partilha de conhecimentos.

À Joana Santos tenho que agradecer a boa-disposição, a amizade e pela partilha de conhecimentos estatísticos entre muitos outros.

Aos meus amigos agradeço a boa-disposição, a ajuda e o apoio constante. Sem vocês este percurso não teria sido tão fácil. Obrigado pela amizade.

À minha família agradeço o apoio incondicional e por nunca me deixar desistir dos meus sonhos.

À Ana Rita Santos, agradeço imensamente toda a ajuda dispensada durante este longo percurso, quer pessoal, quer profissional, a paciência e toda a energia positiva. Obrigado pelas palavras de confiança. Contigo, as coisas tornam-se mais fáceis e mais felizes!

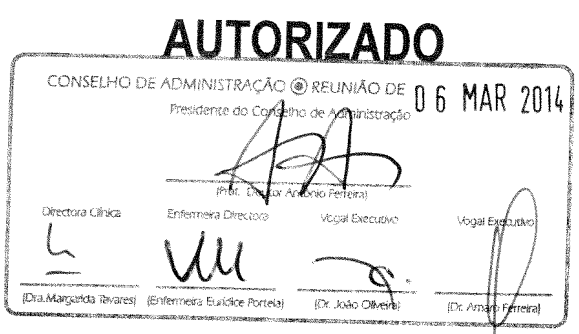
# Parecer da Comissão de Ética

CES 138-13

**Exmo. Senhor**

**Presidente do Conselho de Administração do**

**Centro Hospitalar de S. João – EPE**




**Assunto:** Pedido de autorização para realização de estudo/projecto de investigação

**Nome do Investigador Principal**: Amílcar José Garcia Cordeiro

**Título do projecto de investigação**:

RELEVÂNCIA DO IMPRINTING GENÓMICO NO CRESCIMENTO INTRAUTERINO HUMANO - Estudo da expressão dos genes imprinted IGF2, PHLDA2, CDKN1 e KCNQ1 na restrição de crescimento fetal

Pretendendo realizar no(s) Serviço(s) de Genética do Centro Hospitalar de S. João – EPE o estudo/projecto de investigação em epígrafe, solicito a V. Exa., na qualidade de Investigador/Promotor, autorização para a sua efectivação.

Para o efeito, anexa toda a documentação referida no dossier da Comissão de Ética do Centro Hospitalar de S. João respeitante a estudos/projectos de investigação, à qual endereçou pedido de apreciação e parecer.

Com os melhores cumprimentos.

Porto, 12 / Abril / 2013

O INVESTIGADOR/PROMOTOR

## 7. SEGURO

a. *Este estudo/projecto de investigação prevê intervenção clínica que implique a existência de um seguro para os participantes?*

SIM ☐ (Se sim, junte, por favor, cópia da Apólice de Seguro respectiva)
NÃO ☐
NÃO APLICÁVEL ☒

## 8. TERMO DE RESPONSABILIDADE

Eu, Amilcar José Garcia Cordeiro, abaixo-assinado, na qualidade de Investigador Principal, declaro por minha honra que as informações prestadas neste questionário são verdadeiras. Mais declaro que, durante o estudo, serão respeitadas as recomendações constantes da Declaração de Helsínquia (com as emendas de Tóquio 1975, Veneza 1983, Hong-Kong 1989, Somerset West 1996 e Edimburgo 2000) e da Organização Mundial da Saúde, no que se refere à experimentação que envolve seres humanos. Aceito, também, a recomendação da CES de que o recrutamento para este estudo se fará junto de doentes que não tenham participado em outro estudo no decurso do actual internamento ou da mesma consulta.

Porto, 10 / Abril / 2013

Amilcar Cordeiro
O Investigador Principal

A Comissão de Ética para a Saúde tendo aprovado o parecer do Relator, aguarda que o Investigador/Promotor esclareça as questões nele enunciadas para que possa emitir parecer definitivo.
2013-04-22
Prof. Doutor Filipe Almeida
Presidente da Comissão de Ética

*PARECER DA COMISSÃO DE ÉTICA PARA A SAÚDE DO CENTRO HOSPITALAR DE S. JOÃO*

emitido na reunião plenária da CES de / /

Considerando que foram cumpridos os esclarecimentos e as alterações efectuadas pela equipa de investigação

A Comissão de Ética para a Saúde APROVA por unanimidade o parecer do Relator, pelo que nada tem a opor à realização deste projecto de investigação.

2014-01-14
Prof. Doutor Filipe Almeida
Presidente da Comissão de Ética

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Medicine - Gynecology | Journal of Assisted Reproduction and Genetics

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Editor-in-Chief: David F. Albertini

ISSN: 1058-0468 (print version)
ISSN: 1573-7330 (electronic version)

Journal no. 10815

Instructions for Authors

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Use the equation editor or MathType for equations.

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LaTeX macro package (zip, 182 kB)

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1. Negotiation research spans many disciplines [3].
2. This result was later contradicted by Becker and Seligman [5].
3. This effect has been widely studied [1-3, 7].

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Book

Blenkinsopp A, Paxton P. Symptoms in the pharmacy: a guide to the management of common illness. 3rd ed. Oxford: Blackwell Science; 1998.

Book chapter

Wyllie AH, Kerr JFR, Currie AR. Cell death: the significance of apoptosis. In: Bourne GH, Danielli JF, Jeon KW, editors. International review of cytology. London: Academic; 1980. pp. 251–306.

Online document

Doe J. Title of subordinate document. In: The dictionary of substances and their effects. Royal Society of Chemistry. 1999. http://www.rsc.org/dose/title of subordinate document. Accessed 15 Jan 1999.

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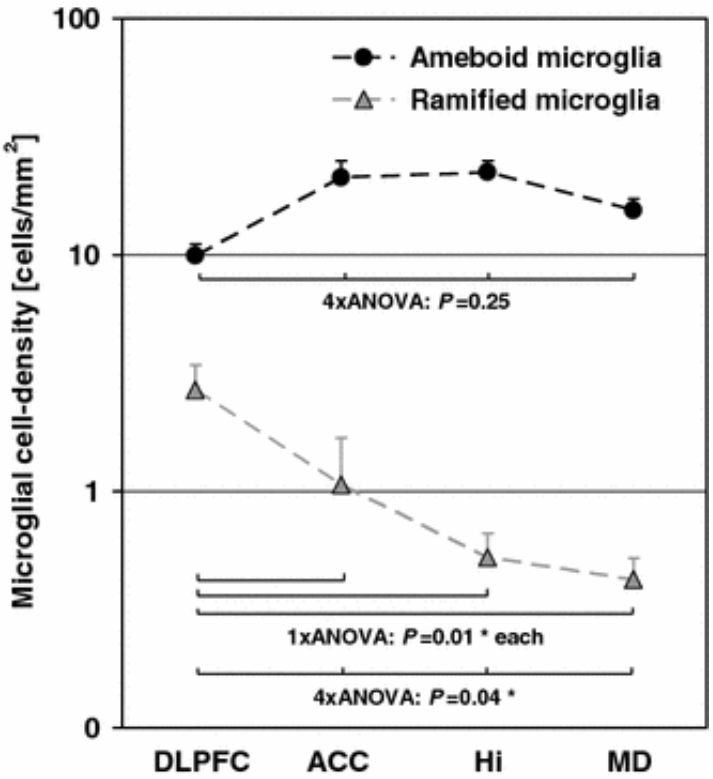


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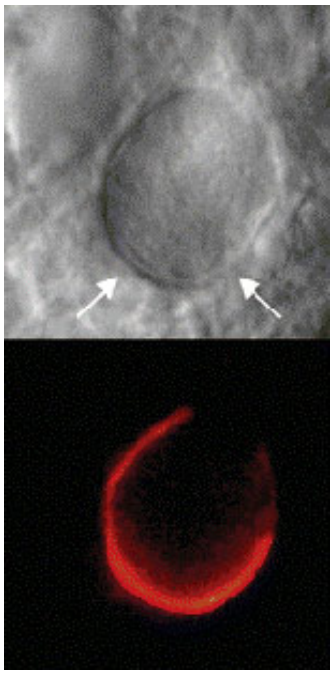

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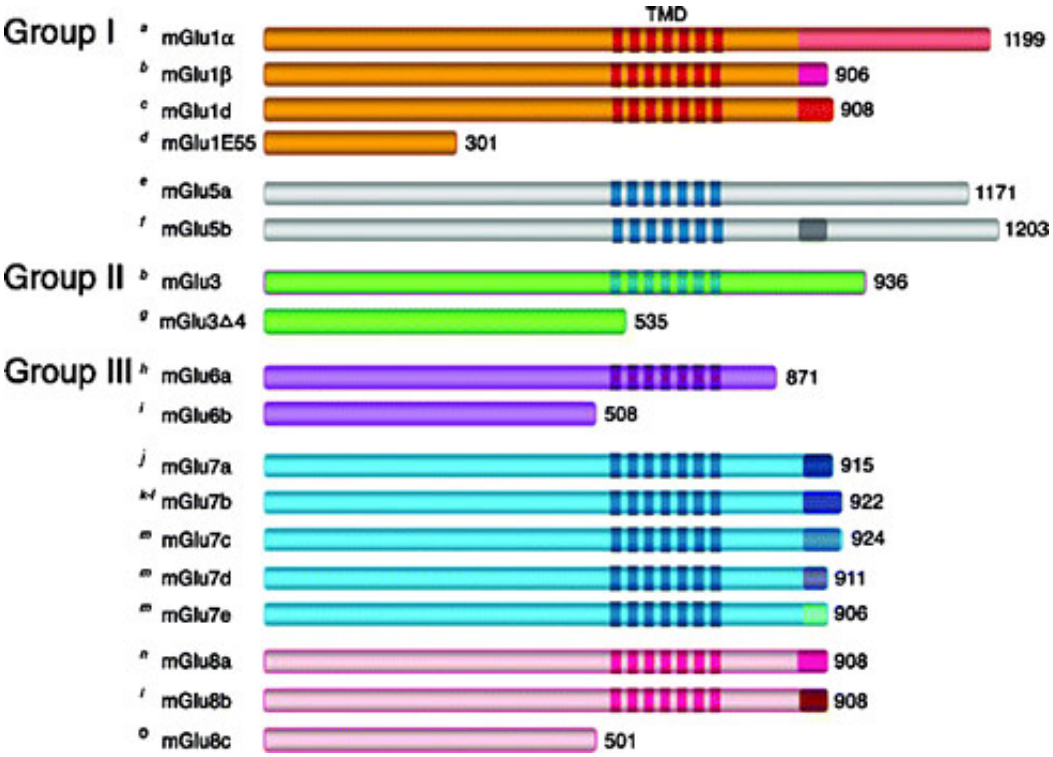


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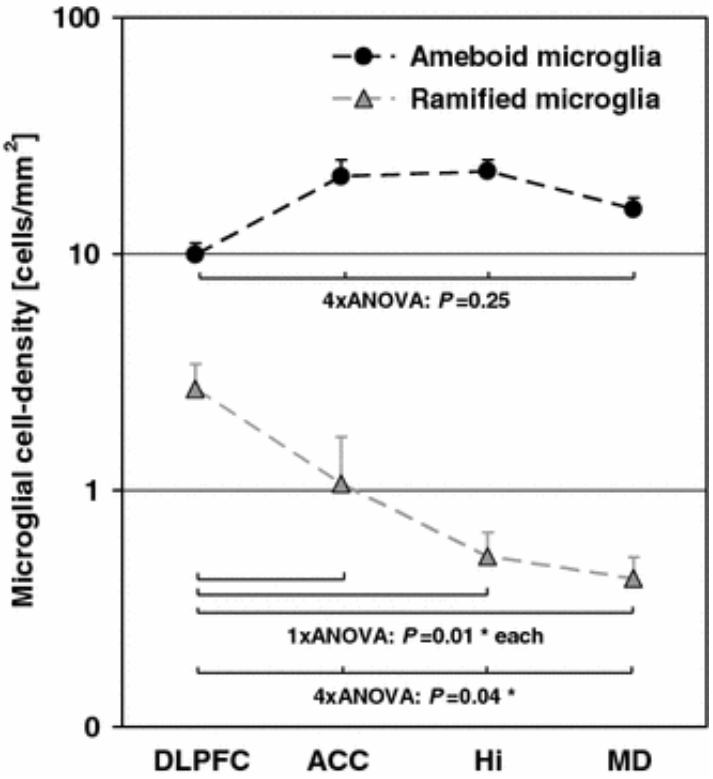


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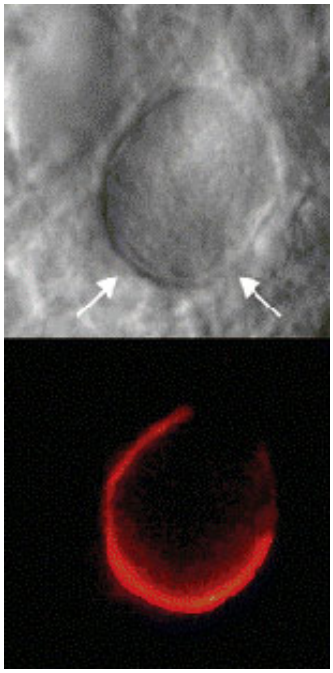

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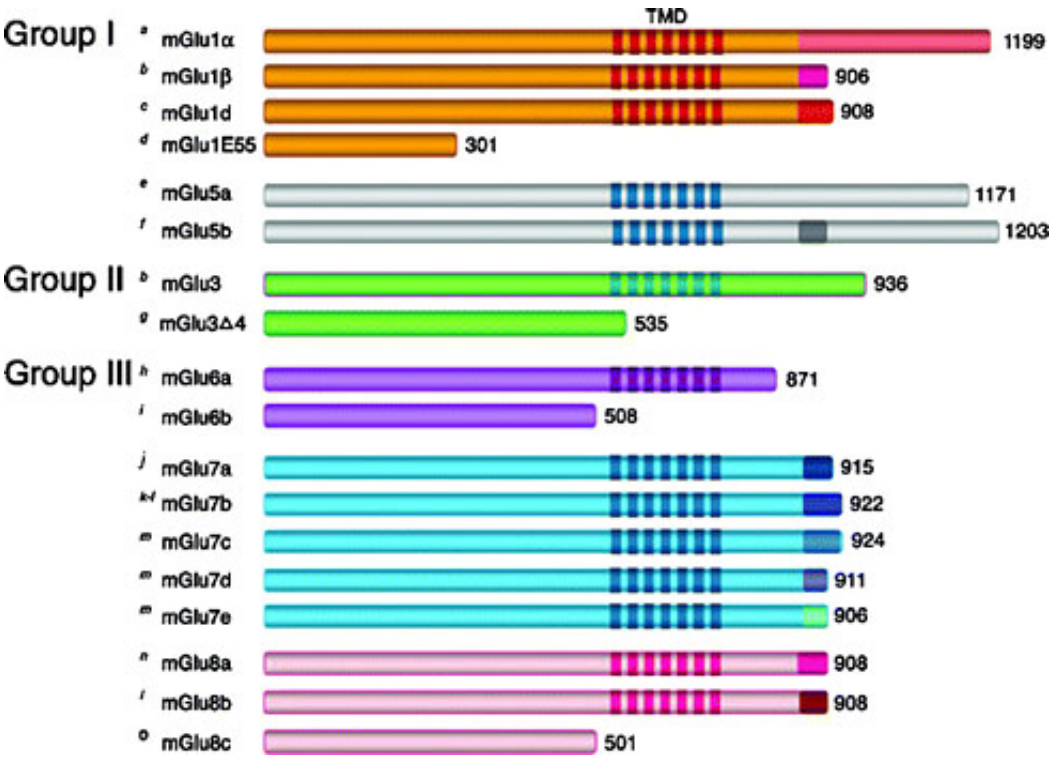


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