# METHYLATION OF THE *C19MC* microRNA LOCUS IN THE PLACENTA: ASSOCIATION WITH MATERNAL AND CHILHOOD BODY SIZE

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**Running title**: *C19MC* methylation and offspring's size

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## ABSTRACT

**Objectives:** To study DNA methylation at the *C19MC* locus in the placenta and its association with: 1) parental body size, 2) transmission of haplotypes for the *C19MC* rs55765443 SNP, 3) offspring's body size and/or body composition at birth and in childhood.

**Subjects and methods:** Seventy-two pregnant women-infant pairs and 63 fathers were included in the study. Weight and height of mothers, fathers and newborns were registered during pregnancy or at birth (n=72). Placental DNA methylation at the *C19MC* imprinting control region (ICR) was quantified by bisulfite pyrosequencing. Genotyping of the SNP was performed using restriction fragment length polymorphisms. The children's body size and composition were reassessed at age 6 years (n=32).

**Results:** Lower levels of placental *C19MC* methylation were associated with increased body size of the mother, specifically with higher pre-gestational and pre-delivery weights and height (β from –0.294 to –0.371; $R^2$ from 0.04 to 0.10 and all p<0.019), and with higher weight, height, waist and hip circumferences, and fat mass of the child (β from –0.428 to –0.552; $R^2$ from 0.33 to 0.56 and all p<0.009). Parental transmission of the SNP did not correlate with an altered placental methylation status at the *C19MC* ICR.

**Conclusions:** Increased maternal size is associated with reduced placental *C19MC* methylation, which, in turn, relate to larger body size of the child.


**Keywords:** maternal size, DNA methylation, programming, rs55765443.

## INTRODUCTION

Genomic imprinting is a complex epigenetically-regulated phenomenon by which some genes become mono-allelically expressed in a parent-of-origin specific manner.[1, 2] Imprinted genes play essential roles in prenatal growth of the embryo and/or placenta functions.[3-6]

In the human placenta, the chromosome 19 microRNA cluster (*C19MC*) is imprinted, and is expressed exclusively from the paternally inherited allele as confirmed by the genotyping of a single nucleotide polymorphism (SNP: G or T, rs55765443) mapping upstream the most-5' microRNA transcribed by *C19MC*.[7] This primate-specific microRNA cluster spans ~100 kb and produces 56 mature microRNAs.[8, 9] The cluster is governed by the DNA methylation status of an imprinting control region (ICR) located about 17.6 kb upstream the microRNA cluster.[7, 10] *C19MC*, which is expressed almost exclusively in placenta,[11] is believed to play important roles in the regulation of cellular differentiation, trophoblast migration and immunomodulation during pregnancy.[12, 13] Altered expression of *C19MC* has been reported in gestational hypertension, preeclampsia and fetal growth restriction.[12, 13]

Fetal growth and postnatal development, which are critical processes of life, are regulated by genetic and epigenetic factors. SNPs located within or in the vicinity of imprinted genes correlate with fetal growth characteristics in a parent-of-origin manner.[14, 15] Non-genetic variation or pathological disruption of DNA methylation marks in several imprinted loci, including *IGF2-H19*, *GNAS* or *DLK1-DIO3,* have been consistently related to changes in pre- and postnatal growth as well.[16-19]

Currently, there are no reports of parental factors related to placental *C19MC* methylation variation, nor about the impact of *C19MC* differential methylation on the offspring's development. Here, we examined for the first time the placental *C19MC* DNA methylation levels and their association with 1) parental weight and height, 2) parental transmission of haplotypes within a SNP (rs55765443) in *C19MC*, 3) postnatal growth and body composition of the offspring at birth and in childhood, and 4) gene expression levels of representative *C19MC* miRNAs.

## METHODS

*Study population and ethics*

The study population included 72 pregnant Caucasian women who delivered healthy infants, and 63 of the respective fathers who also accepted to participate in this longitudinal cohort study (Table 1). The subjects were recruited during the first trimester of pregnancy among those seen within a setting of prenatal primary care in l'Alt Empordà and Girona (North-eastern Spain) from 2008 to 2010. Information on pregnancy, labor and delivery characteristics was retrieved from standardized medical records. Pregnancies resulting from assisted reproductive technology (ART) were excluded because ART may perturb imprinting.[20] Women with major medical, surgical or obstetrical complications, including multiple pregnancies, hypertension, gestational diabetes or preeclampsia, and fetal growth restriction, malformations or asphyxia were also excluded.

The protocol was approved by the Institutional Review Board of Dr. Josep Trueta Hospital (Reference number: 2013132; Approval date: November 24th 2014) and informed written consent was obtained from all parents.

*Anthropometric assessments*

Father's weight and height were measured at inclusion without shoes and wearing light clothes; maternal weight and height were assessed similarly at each trimester of gestation. Body-mass index (BMI) was calculated as weight divided by height squared (Kg/m$^2$).

All infants were born at term of pregnancy. After delivery, weight and length were measured using a calibrated scale and a measuring board. Gestational age- and sex-adjusted z-scores for birth weight and length were calculated using regional norms.[21] From the children included at birth, those whose parents agreed that they could participate further in the study (n=32) were followed-up at the age of 6 years. Their characteristics at birth did not differ from those who did not participate in the follow-up study. Weight was measured on a calibrated scale wearing light clothes, and height was measured with a Harpenden stadiometer without shoes. BMI and age- and sex-adjusted z-scores were calculated as above. Waist circumference was measured in the supine position at the umbilical level. Hip circumference was measured at the widest part, at the level of the greater trochanters.

*Fat measurements*

Fat mass was assessed by bioelectric impedance (Hydra Bioimpedance Analyzer 4200; Xitron Technologies, San Diego CA), at the age of 6 years, as previously described in prepubertal children.[22]

Visceral fat was estimated as described by Hirooka et al[23] using high-resolution ultrasonography (MyLabTM25, Esaote, Italy) in a transverse abdominal scan with a convex 3-3.5 MHz transducer, with minimal pressure applied to prevent compression of the fat layers. Measurement of visceral fat by ultrasound correlates well with that measured by computed tomography.[23, 24] All images were obtained with the subject in supine position at the end of a normal exhalation and performed by the same observer. The average of three measurements was used for all sites; the intra-subject coefficient of variation was <6%.

*Sample collection*

Blood samples were drawn from both progenitors in EDTA tubes at the initial visit. Samples were centrifuged at 2000 x g for 15 minutes at 4ºC and total leukocytes were harvested. The placentas were collected immediately after childbirth. Three cuboidal biopsies

(1 $cm^3$) containing placental villous tissue, were dissected from the non-membranous surface (maternal side) of placentas after removing the decidua (outermost layer) midway between the umbilical cord and the placental margin. The same location was used when sampling all placentas to reduce interplacental variability. All samples were stored at -80ºC.

*DNA methylation analysis*

Quantitative DNA methylation analysis was performed by pyrosequencing of bisulphite-treated DNA.[25] Genomic DNA was extracted from placentas using the Gentra PureGene tissue kit (Qiagen, Germany). Sodium bisulfite conversion of 500 ng of DNA was performed using the EZ DNA Methylation-Gold kit (Zymo Research, Irvine CA). Bisulphite-treated DNA (20 ng) was amplified with 0.3 µM of forward and biotinylated reverse primers (Supplementary Table S1). Reactions were performed in 1X Taq GOLD buffer adjusted to 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 1 U of Taq GOLD polymerase (Qiagen) in a total volume of 35 µl. PCR cycling steps were: 15 min at 94°C followed by 40 cycles of 20 s at 94°C, 30 s at 58.6ºC and 1 min at 72°C, with a final extension of 10 min at 72°C. PCR product was rendered single-stranded[25] and 4 pmol of the sequencing primer (Supplementary Table S1) were added following pyrosequencing in PyroMark Q96 ID and Q96 MD instruments (Qiagen). Raw data were analyzed using the Q-CpG software (V.1.0.9, Biotage AB), which calculates the ratio of converted Cs (Ts) to unconverted Cs at each CpG, giving the percentage of methylation. All reactions were run in duplicates. We analyzed 5 consecutive CpG dinucleotides located within the *C19MC* ICR (chr19:53648001-53648160 from UCSC Genome Browser, Human Dec. 2013 (GRCh38/hg38) Assembly, Supplementary Figure S1A). The overall DNA methylation level across the *C19MC* ICR was defined as the average of methylation at all 5 CpG sites.

*Genotyping (Restriction Fragment Length Polymorphisms, RFLPs)*

Mother-father-child trios were genotyped in order to study the paternal and maternal transmission of rs55765443 SNP alleles to the child. To assess parental genotype, DNA was extracted from parental blood leukocytes using the Gentra PureGene blood kit (Qiagen). For fetal genotyping, DNA was extracted from placentas as described above. DNA (10 ng) was amplified in a 10 µl reaction with 1x $NH_4$ buffer supplemented with 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 U BIOTAQ DNA Polymerase (Bioline, Memphis TN) and 0.6 pmol/µl of each primer. PCR primers (chr19:53665044; UCSC Genome Browser, Human Dec. 2013 (GRCh38/hg38) Assembly) were: forward 5'-TGTGGCCAGACTTTAATCCA-3' and reverse 5'-TTGGAGATTTTAGGGGGAGTC-3'. PCR conditions were: 94ºC for 5 min; 20 cycles of 94ºC 45 s, 64ºC 45 s (decreasing 0.5ºC each cycle) and 72ºC 45s; 15 cycles of 94ºC 45 s, 54ºC 45 s and 72ºC 45s; and 72ºC for 10 min. PCR product (207 bp) was then digested with 1 U of *Bso*BI restriction enzyme (New England Biolabs, Ipswich Massachusetts) at 37ºC for 16 hours. The fragments were visualized by electrophoresis through a 2% (w/v) agarose gel. The different genotypes were assessed according to the size and pattern of distribution of the fragments (Homozygous T: 1 band 207 bp; Heterozygous: 3 bands of 207, 152 and 55 bp; Homozygous G: 2 bands of 152 and 55 bp). Mother-father-child trios that were all heterozygous were uninformative for imputing parental allelic transmission, thus the final number of samples used in the analysis was 53 trios. All SNP genotypes were shown to be consistent with Hardy–Weinberg equilibrium ($\chi^2$ test p=0.900).

*Gene expression analysis by Real Time-PCR*

Total RNA was extracted and retrotranscribed using the RNeasy mini kit (Qiagen) and the Megaplex Human RT Primers (Pool A v2.1) with MultiScribe Reverse Transcriptase (Thermofisher Scientific, Waltham, MA). The following TaqMan Gene Expression assays (Thermofisher Scientific) were used to amplify the cDNA: miR-517a (Ref#002402), miR-517c (Ref#001153), miR-520c (Ref#002389), miR-520g (Ref#001121) and the endogenous controls miR-523 (Ref#002386), miR-532 (Ref#001518) and miR-425-5p0 (Ref#425-5p).[26] Reactions were run on a LightCycler 480 Real-Time PCR System (Roche Diagnostics, Rotkreuz, Switzerland), using the default cycling conditions. Relative mRNA levels were calculated according to the $2^{-\Delta CT}$ method.

*Statistics*

Statistical analyses were performed using the SPSS 22.0 package (SPSS Inc). Non-normally distributed data was log-transformed to improve symmetry. The relationship between DNA methylation levels and both anthropometric parameters and gene expression was tested by Pearson correlation followed by multiple regression analysis using the enter method to adjust for possible confounding variables (maternal pre-gestational and gestational weight, maternal height, gestational age, birth weight or length, and child's sex, age and BMI). Differences in DNA methylation levels among haplotypes were examined by unpaired Student's T-test. Accepting an alpha risk of 0.05 in a bilateral contrast, the study has an 80% power to detect a significant Pearson correlation coefficient of at least 0.5 between variables, and a difference of at least 5 units in DNA methylation between groups assuming a standard deviation (SD) of 5 units (GRANMO, IMIM, version 7.12). When analyzing the percentage of methylation of each CpG, the statistical significance was set at 0.0125 after applying the

Bonferroni correction for subgroup analyses (0.05/4 subgroup analyses: maternal characteristics, paternal characteristics, data at birth and childhood data).

## RESULTS

*Subjects*

Table 1 shows the clinical variables in the parents and their children, at birth and at 6 years of age. Maternal anthropometric variables (height, pre-gestational and predelivery weights, and pregestational BMI) associated with offspring's variables (weight, height and BMI) at birth and 6 years of age (Supplementary Table S2).

*DNA Methylation levels at C19MC in placental tissue*

DNA methylation levels of the 5 studied CpGs within the ICR region of *C19MC* (Supplementary Figure S1A) were assessed by pyrosequencing. The percentage of DNA methylation in placenta for each CpG and the average methylation for all CpGs are shown in Supplementary Figure S1B. Average methylation levels of the combined CpGs at *C19MC* ICR ranged from 26 to 56% with mean and SD values of 42.7 ± 7.9 %. The methylation levels at the different CpGs analyzed were highly concordant. This indicates that methylation was affected similarly across the entire genomic region.

*Higher maternal size correlates with lower C19MC methylation in the placenta*

Firstly, we aimed to study the relationship between methylation levels at the *C19MC* ICR and parental phenotype. Higher maternal pre-gestational weight ($r=-0.316$, $p=0.007$), height ($r=-0.291$, $p=0.013$) and BMI ($r=-0.237$, $p=0.045$), as well as pre-delivery weight ($r=-0.376$, $p=0.002$), associated with lower mean methylation levels at the placental *C19MC* ICR (Figure 1 A-C and Supplementary Table S3). Paternal anthropometric variables were not related to methylation levels within the placental *C19MC* cluster (Supplementary Table S3).

Following correction for multiple testing, maternal pre-gestational weight, height and pre-delivery weight remained significantly associated with mean methylation at *C19MC* ICR (all p≤0.01). Maternal pre-gestational weight (p=0.011), height (p=0.019) and pre-delivery weight (p=0.003) independently explained mean placental *C19MC* methylation levels in multivariate linear models after adjusting for the following confounding variables: maternal age, gestational age and child's sex, with model $R^2$ of 0.05, 0.04 and 0.10, respectively (Supplementary Table S4).

*Parentally transmitted alleles for the rs55765443 SNP and placental C19MC DNA methylation*

We further assessed whether placental *C19MC* ICR methylation levels were related to the parental transmission of the rs55765443 SNP within the *C19MC* cluster. Maternally transmitted haplotypes had no significant effect on placental *C19MC* methylation levels (Supplementary Figure S2A and Table S5).

Fetal inheritance of the paternal T allele was associated with lower methylation levels (39.8 ± 2.0 %) when compared with the G allele (47.2 ± 2.0 %) only at CpG5 of *C19MC ICR* (p=0.05, Supplementary Table S5). As for mean *C19MC* ICR methylation levels, the difference between T (41.0 ± 1.4 %) and G alleles (45.3 ± 1.6 %, Supplementary Figure 2B and Table S5) approached significance (p=0.059). However, none of these associations remained significant after correction for multiple testing.

*Lower placental C19MC methylation levels associates with bigger offspring's size at 6 years of age*

Next, we examined the consequences of the differential DNA methylation at the placental *C19MC* ICR on the offspring at birth and at age 6 years. At birth, no statistically significant relationships between newborns' anthropometric variables and *C19MC* methylation levels were observed (Supplementary Table S6). However, lower mean methylation levels at placental *C19MC* ICR correlated with larger children's size and fat abundance at age 6 years [weight ($p \leq 0.001$), height ($p=0.001$), BMI ($p=0.017$), waist ($p=0.003$), hip ($p=0.003$), fat mass ($p=0.005$) and visceral fat ($p=0.009$); Figure 2 and Supplementary Table S6].

The correlations between methylation at *C19MC* ICR and weight, height, waist circumference, hip circumference, fat mass or visceral fat in the offspring remained significant after correction for multiple testing (all $p \leq 0.0125$). Placental *C19MC* ICR methylation levels also remained independently associated with children's anthropometric variables (weight, height, waist and hip) after adjusting for confounding variables, including maternal weight or height, in general linear models (Table 2). The association of *C19MC* ICR methylation with fat mass, but not that with visceral fat, was also independent of confounding variables (Table 2).

*Placental C19MC ICR methylation levels and gene expression*

Finally, we investigated the potential link between *C19MC* ICR methylation and gene expression in the placental samples. Due to the complexity of this locus, which is transcribed as a single pri-miRNA and post-transcriptionally processed to generate numerous miRNAs with a wide range of expression levels,[27, 28] we quantified the expression of representative miRNAs (miR-517a, miR-517c, miR-520c and miR-520g) in 56 placental samples. These miRNAs were chosen in order to have diverse expression levels (high, medium, low) and a wide coverage across the cluster. Median expression of the 4 analyzed miRNAs was used in order to approximate the gene expression levels of *C19MC*. In a bivariate correlation analysis, increased *C19MC* ICR methylation associated with reduced median miRNA expression (r=

–0.267, p=0.047; Figure 3). This finding suggests that ICR methylation negatively affects miRNA expression across the locus.

## DISCUSSION

Although altered expression of imprinted gene clusters has been linked to postnatal growth disorders,[29] evidence that the placental imprinted *C19MC* cluster may play a role in this process is missing. Here, we show for the first time that, in the placenta, the level of methylation at the *C19MC* ICR is linked to maternal weight and height, and is ultimately related to the offspring's body size and body composition in childhood.

Interestingly, placental *C19MC* ICR methylation associated with the offspring's anthropometry, independently of maternal weight and height. The association found in our cohort, between maternal weight/height and offspring's weight/height, may be explained at least partially by placental *C19MC* ICR methylation levels. Thus, besides genetic factors,[30] the offspring's size may be significantly modulated by epigenetic factors at early stages of life. More generally, and in agreement with our finding, gene variants associated with height or BMI have been found to account for only around 20% of the heritable phenotypic variation.[31, 32]

Different studies have shown that maternal size correlates with the offspring's growth and body composition.[33-35] Our results suggest a possible role for placental *C19MC* ICR methylation in the modulation of childhood growth. Similarly, placental DNA methylation at the *IGF2-H19*, *GNAS* and *DLK1-DIO3* imprinted loci has been linked to pre and postnatal growth characteristics.[16, 18, 19] Moreover, our results link placental *C19MC* ICR methylation to the body composition of the offspring in childhood. Interestingly, maternal obesity has been suggested to alter adipocyte commitment and differentiation in the offspring via an epigenetic mechanism as well.[36] Our findings suggest that maternal size and offspring fat mass at 6 years of age may, at least in part, be linked to *C19MC* methylation.

It has been shown that maternal factors, such as nutrient supplementation or alcohol intake, can alter DNA methylation patterns of imprinted genes with paternal expression, for instance at *MEST*, *PLAGL1* or *IGF2*.[37-40] It is therefore plausible that maternal body weight or height, may also influence placental methylation as well. In principle, the maternal allele is virtually 100% methylated at *C19MC*, as opposed to the paternal allele, which is 0% methylated. However, we found *C19MC* ICR global methylation to be less than 50% in most cases. This suggested that the maternal allele had partially lost its imprinting marks, thus allowing limited maternal expression and probably leading to functional consequences due to the high dosage sensitivity of imprinted gene expression.[41]

In contrast to pre-gestational maternal weight, gestational weight gain cannot be directly linked to placental *C19MC* ICR methylation. Indeed, previous studies have suggested that nutrition throughout gestation does not contribute to the epigenetic reprogramming of the ICRs of *GNAS*, *GRB10*, *KCNQ1OT1/CDKN1C* and *H19/IGF2* loci.[42, 43] These findings could be related to the imprinted condition of the studied gene clusters. DNA methylation at ICRs of imprinted genes is acquired during gametogenesis, thus establishing germline-derived differentially methylated regions, and is refractory to the genome-wide methylation reprogramming that occurs in the embryo after fertilization.[20, 29, 44] Accordingly, maternal weight during oogenesis and early embryogenesis, rather than gestational weight gain, could affect the DNA methylation at the imprinted *C19MC* ICR.

DNA methylation levels can also be modified by the presence of specific SNPs.[45] SNPs neighboring CpG islands, such as rs55765443, can lead to allele-specific DNA methylation changes, usually in a cis-acting mechanism.[46] In this line, several obesity-associated SNPs have been reported to be associated with methylation levels at proximal CpG sites.[47] In the *C19MC* cluster, the maternal allele is methylated by a specific imprint acquired in oocytes,[7] thus it would be plausible that a SNP in the maternally transmitted allele could affect *C19MC* ICR methylation levels. We therefore studied the relationship of the maternal and paternal alleles for the rs55765443 SNP with methylation levels. While no association was evident for transmission of the maternal allele and *C19MC* ICR methylation levels, we found a tendency for the paternally transmitted allele to be associated with altered methylation levels, which was not confirmed in adjusted analyses. We cannot rule out the possibility that other SNPs located close to this region could be linked to altered methylation levels of the *C19MC* ICR.

Epigenetic variations at placental *C19MC* may be associated with concordant levels of change in gene expression that could explain the observed link between the cluster and offspring's anthropometry. In fact, *C19MC* microRNAs can traffic among placental, maternal and fetal compartments,[48] feasibly influencing fetal programming and thus the developmental trajectory. Computational and bioinformatic analyses have predicted that *C19MC* miRNAs participate in reproduction, development, and differentiation,[49] including stem cell self-renewal and pluripotency by controlling G1-S transition and apoptosis signaling.[50] Indeed, members of the *C19MC* cluster have been shown to regulate cellular reprogramming, enhance proliferation and suppress apoptosis.[51] Regarding our results, a possible hypothesis is that differential methylation at the *C19MC* cluster may cause molecular changes in fetal tissues, probably in stem cells, through variations in microRNAs levels. *C19MC* microRNAs may thus target and prime specific fetal tissues, such as adipose tissue, for enhanced proliferation later in life. This hypothesis could not be tested in our study. Instead, we analyzed the expression levels of representative *C19MC* miRNAs in placental tissue and found that methylation levels in this locus associated with miRNA expression levels. Although the molecular changes may be rather fast, the anthropometric changes (weight, height or fat mass) could need more time to be revealed, and thus they may not be seen until childhood, explaining the lack of association with infants' anthropometry.

We acknowledge some study limitations. The specific role for the rs55765443 paternal haplotype on *C19MC* methylation, as well as the interaction between the paternally transmitted allele and maternal size as modifiers of the placental *C19MC* ICR methylation levels, need to be addressed in additional studies. A next step should also include the analysis in fetal tissues of gene expression levels of the 56 microRNAs encoded by *C19MC* cluster, which was beyond the scope of the current study. Finally, other factors such as diet or other environmental factors were not included in the current analysis but should be considered for further studies as possible confounders.

The strengths of our study include the follow-up data on the offspring. Moreover, due to the crucial role of imprinted genes in early life development, the significance of our study is warranted, since elucidating the factors that affect their epigenetic regulation has important implications for understanding the causes of human health and disease, and may help to establish protocols for early detection/prevention of adult diseases. Finally, the use of placenta to predict complications later in life would have obvious advantages, as it is an easily available tissue that can be sampled non-invasively.

In summary, maternal size associates with the percentage of methylation within the placental *C19MC* ICR, and such methylation levels are related to offspring's size and body composition at age 6 years. Increased maternal size may reduce placental *C19MC* methylation, in turn leading to larger size of the offspring in childhood. These results may help to establish protocols for early detection/prevention of childhood/adulthood diseases related to body size and composition.

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The authors declare that they have no conflicts of interest.

Supplementary information is available at International Journal of Obesity's website.

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**FIGURE LEGENDS**

**Figure 1. Higher maternal size correlated with lower *C19MC* methylation in placenta. (A)** Scatter plot of pre-gestational maternal weight and *C19MC* ICR mean methylation. **(B)** Scatter plot of maternal height and *C19MC* ICR mean methylation. **(C)** Scatter plot of pre-delivery maternal weight and *C19MC* ICR mean methylation. Pearson correlation statistics are shown within each box.

**Figure 2. Lower placental *C19MC* ICR methylation associated with greater offspring's size and adiposity at 6 years of age.** Scatter plots showing the correlation of placental *C19MC* ICR methylation levels with offspring's weight z-score **(A)**, height z-score **(B)**, waist circumference **(C)**, hip circumference **(D)**, fat mass **(E)** and visceral fat volume **(F)** at 6 years of age. Pearson correlation statistics are shown in the boxes.

**Figure 3. Placental methylation levels at the C19MC ICR and gene expression.** Scatter plot showing the correlation of placental *C19MC* ICR methylation levels with median *C19MC* gene expression (n=56). Pearson correlation statistics are shown in the box.

June 17th, 2019

**Dr. Ian Macdonald**

**Editor**

**International Journal of Obesity**

**RE: Manuscript ID 2019IJO00131**

Dear Dr. Ian Macdonald,

Regarding the manuscript referenced above, we appreciate the interest that the editors and reviewers have taken in our manuscript and the constructive criticism they have given.

We have addressed the concerns of the reviewers. These changes have clearly improved our manuscript and a revised version has been uploaded on the *International Journal of Obesity's* website. Changes to the text in the manuscript are highlighted in red.

In addition to making the changes described above, we have also included a point-by-point response to the reviewers.

We hope that the revised version of the manuscript is acceptable for publication in the *International Journal of Obesity*.

Sincerely yours,

**Abel López-Bermejo, MD**
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# REVIEWER'S COMMENTS

## Associate Editor (Comments to the Author):

Please change the term "gender" (societal role) to "sex" if biologic category is being defined.

"Gender" has been replaced by "sex" throughout the manuscript and tables/figures as it defines a biologic category.

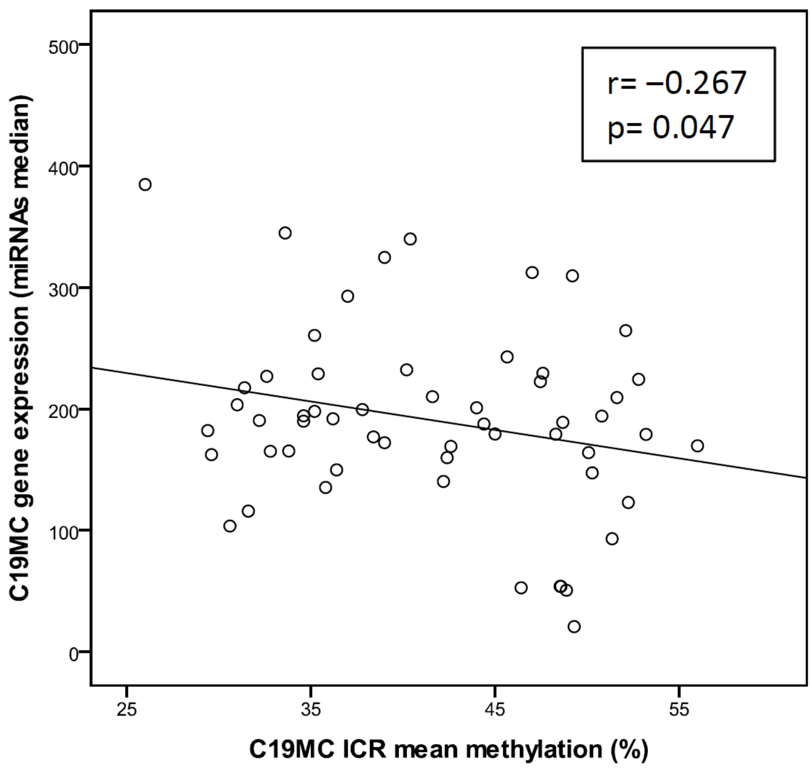
## Reviewer #1 (Comments to the Author):

In this study Prats-Puig et al describe a series of studies looking at the methylation of the C19MC in the placenta and the association of this methylation with body size and composition of the child at birth and 6 years of age as well as in the body size of the parents. They quantify DNA methylation of 5 CpGs in the ICR for the cluster and show that the methylation of these CpGs is concordant. They then show that methylation of the ICR is inversely associated with maternal size but there are no associations with paternal size. Comparison of the methylation of the ICR with offspring phenotype did not show any associations with birth measurements but did show associations with the anthropomorphic measurements in the subset analysed at 6 years of age.

Overall, I think that this is a nice study that is clearly presented. My major criticism is based around the question of whether it is possible to quantify the expression of at least one of the C19MC miRNAs in the placental tissue that has been collected. This is not to look at the expression in fetal tissues which obviously can't be done for the individuals included in this study (and which the authors say is beyond the scope of this study). The reason for this is that it would give them a more direct link to this set of miRNAs rather than other genes in this locus that are paternally expressed. It would also confirm that methylation of this locus is linked to expression levels of the miRNA (at least in the placenta). Obviously it can only be done if the samples are available in a suitable format.

We agree with the reviewer's point. In order to address this issue, we have quantified the expression levels of representative miRNAs (miR-517a, miR-517c, miR-520c and miR-520g) in 56 cases with available placental samples. These miRNAs have been chosen in order to have a wide coverage within the cluster and a wide range of expression levels (high, medium, low) according to previous data [Donker RB et al. The expression profile of C19MC microRNAs in primary human trophoblast cells and exosomes. Mol Hum Reprod 2012 Aug 28(8) 417-424]. Due to the complexity of this locus, which is transcribed as a single pri-miRNA and post-transcriptionally processed to generate numerous miRNAs [Bellemer CJ et al. Microprocessor dynamics and interactions at endogenous imprinted C19MC microRNA genes. Cell Sci. 2012 Jun 1;125(Pt 11):2709-20], we calculated the median expression of the 4 analyzed miRNAs in order to estimate the global gene expression of *C19MC*.

In a bivariate correlation analysis, *C19MC* ICR methylation levels associated with median miRNA expression (r= –0.267, p=0.047; Figure 3).



According to this, the manuscript has been mainly modified as follows:

MATERIALS AND METHODS:

*"Gene expression by Real Time-PCR*

*Total RNA was extracted and retrotranscribed using the RNeasy mini kit (Qiagen) and the Megaplex Human RT Primers (Pool A v2.1) with MultiScribe Reverse Transcriptase (Thermofisher Scientific, Waltham, MA). The following TaqMan Gene Expression assays (Thermofisher Scientific) were used to amplify the cDNA: miR-517a (Ref#002402), miR-517c (Ref#001153), miR-520c (Ref#002389), miR-520g (Ref#001121) and the endogenous controls miR-523 (Ref#002386), miR-532 (Ref#001518) and miR-425-5p0 (Ref#425-5p).26 Reactions were run on a LightCycler 480 Real-Time PCR System (Roche Diagnostics, Rotkreuz, Switzerland), using the default cycling conditions. Relative mRNA levels were calculated according to the 2–ΔCT method."*

RESULTS:

*"Placental C19MC ICR methylation levels and gene expression*

*Finally, we investigated the potential link between C19MC ICR methylation and gene expression, both in placental samples. Due to the complexity of this locus, which is transcribed as a single pri-miRNA and post-transcriptionally processed to generate numerous miRNAs with a wide range of expression levels,27, 28 we quantified the gene expression of representative miRNAs (miR-517a, miR-517c, miR-520c and miR-520g) in 56 placental samples. These miRNAs were chosen in order to have diverse expression levels (high, medium, low) and wide coverage within the cluster. Median expression of the 4 analyzed miRNAs was used in order to estimate* *the global gene expression of C19MC. In a bivariate correlation analysis, C19MC ICR methylation levels associated with median miRNA expression (r= –0.267, p=0.047; Figure 3).*"

My other man point is that the associations at 6 years are from a sub-population of the whole study and indicate either a difference between the effects of the miRNAs at the two ages or the steady accumulation of effect until it is large enough to measure. However, it is also possible that it is an artefact of the subgroup. I accept that there is no difference in the birthweight of those who did or did not come back for the follow-up but it would be useful to confirm that there is no association of the methylation status with birthweight anthropomorphic measurements in the 32 individuals that form the 6 year old group. If such an association did exist in the subgroup it would complicate the interpretation of the data.

Regarding the reviewer's comment, we have studied the association of birth weight/height and methylation in the subpopulation with follow-up (n=32).

The associations have been adjusted for maternal/paternal height or weight in multiple regression analyses. As shown in the table below, *C19MC* ICR methylation was not associated with either sex- and gestational age-adjusted birth weight (Birth weight z-score) or height z-score significantly associated with in the subpopulation with follow-up.

**Table 1.** Multivariate linear models of placental *C19MC ICR* methylation levels and birth weight/height in the follow-up subpopulation (n=32).

| | β | p | Total $R^2$ |
|---|---|---|---|
| **Birth weight z-score** | | | **0.18** |
| *C19MC ICR mean methylation (%)* | -0.180 | 0.341 | |
| *Maternal weight (Kg)* | 0.203 | 0.313 | |
| *Paternal weight (Kg)* | 0.453 | 0.015* | |
| **Birth height z-score** | | | **0.33** |
| *C19MC ICR mean methylation (%)* | -0.231 | 0.145 | |
| *Maternal height (cm)* | 0.133 | 0.443 | |
| *Paternal height (cm)* | 0.592 | 0.001*** | |

**Reviewer #2 (Comments to the Author):**

This paper by Prats-Puig et al, investigate the methylation of the human placental-specific imprinted miRNA cluster C19MC in links with body size in the mother and child. The analysis is focused on the methylation status of 5 CpG located in the Imprinted Control Region of C19MC, evaluated by pyrosequencing. The authors show a series of negative correlation between the placental methylation and maternal body parameters, a link between paternal allelic transmission and methylation level. Lower methylation is associated to increased offspring size and weight at 6 years.

These results are interesting and novel, especially the follow-up of the offspring. The paper is well written and balanced.

I have some minor remarks and questions:

1. Table 1 should be presented as part of the material and methods instead as in the results.

According to the reviewer's suggestions, we have presented Table 1 in the material and methods section:

*"Study population and ethics*

*The study population included 72 pregnant Caucasian women delivering healthy infants, and 63 of the respective fathers who also accepted to participate in this longitudinal cohort study (Table 1)."*

2. Is there an effect of the parity on methylation of the ICR? Even if it is not possible to have a longitudinal analysis (i.e. several children from the same mother) it may be evaluate globally on the various samples. In the same order of ideas, are there samples from dizygotic twins; in

this case how is the methylation of the placentas? What is the link with the growth later, if here are some samples of this kind in the collection?

Regarding the reviewer's point, we have studied whether parity influences *C19MC* ICR methylation levels. As shown in the table below, parity does not influence *C19MC* ICR methylation levels in the placenta.

**Table 2.** Effect of parity on *C19MC* ICR methylation levels.

| Parity | *C19MC* methylation (%) | Student's T test p-value |
|---|---|---|
| Primiparous (n=41) | 42.4 ± 8.0 | **0.583** |
| Multiparous (n=31) | 43.4 ± 8.0 | |

Regarding the other points raised by the reviewer, twin pregnancies were excluded in our study and therefore we cannot provide relevant information to his/her comment.

3. Given the effects found by the authors, would not it be nice to incorporate a discussion in relation with the different papers that identify gene variants linked to height and that explain only very partly the existing variation such as Lango Allen et al (Nature 2010)

Following the reviewer's suggestions, we have included this information in the discussion section:

*"In our study, methylation at placental C19MC ICR associates with offspring's anthropometry independently of maternal weight and height, and thus the association found in our cohort between maternal weight/height and offspring's weight/height may be explained at least partially by placental C19MC ICR methylation. Our results therefore suggest that besides genetic factors,[29] the offspring's size may be also modulated by epigenetic factors at early stages of life. Not surprisingly, Genome-Wide Association Studies have identified gene variants*

*associated with height or BMI that are only able to explain around 20% of the heritable variation.*[30, 31]"

# METHYLATION OF THE *C19MC* microRNA LOCUS IN THE PLACENTA: ASSOCIATION WITH MATERNAL AND CHILHOOD BODY SIZE

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**Running title**: *C19MC* methylation and offspring's size

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**Word count:** Abstract 219; Main Text 3575; 2 Tables and 3 Figures, 8 Supplementary Tables and Figures.

## ABSTRACT

**Objectives:** To study DNA methylation at the *C19MC* locus in the placenta and its association with: 1) parental body size, 2) transmission of haplotypes for the *C19MC* rs55765443 SNP, 3) offspring's body size and/or body composition at birth and in childhood.

**Subjects and methods:** Seventy-two pregnant women-infant pairs and 63 fathers were included in the study. Weight and height of mothers, fathers and newborns were registered during pregnancy or at birth (n=72). Placental DNA methylation at the *C19MC* imprinting control region (ICR) was quantified by bisulfite pyrosequencing. Genotyping of the SNP was performed using restriction fragment length polymorphisms. The children's body size and composition were reassessed at age 6 years (n=32).

**Results:** Lower levels of placental *C19MC* methylation were associated with increased body size of mother, specifically with higher pre-gestational and pre-delivery weights and height of the mother (β from –0.294 to –0.371; $R^2$ from 0.04 to 0.10 and all p<0.019), and with higher weight, height, waist and hip circumferences, and fat mass of the child (β from –0.428 to –0.552; $R^2$ from 0.33 to 0.56 and all p<0.009). Parental transmission of the SNP did not correlate with an altered placental methylation status at the *C19MC* ICR.

**Conclusions:** Increased maternal size is associated with reduced placental *C19MC* methylation, which, in turn, relate to larger body size of the child.


**Keywords:** maternal size, DNA methylation, programming, rs55765443.

## INTRODUCTION

Genomic imprinting is a complex epigenetically-regulated phenomenon by which some genes become mono-allelically expressed in a parent-of-origin specific manner.[1, 2] Imprinted genes play essential roles in prenatal growth of the embryo and/or placenta functions.[3-6]

In the human placenta, the chromosome 19 microRNA cluster (*C19MC*) is imprinted, and is expressed exclusively from the paternally inherited allele as confirmed by the genotyping of a single nucleotide polymorphism (SNP: G or T, rs55765443) mapping upstream the most-5' microRNA transcribed by *C19MC*.[7] This primate-specific microRNA cluster spans ~100 kb and produces 56 mature microRNAs.[8, 9] The cluster is governed by the DNA methylation status of an imprinting control region (ICR) located about 17.6 kb upstream the microRNA cluster.[7, 10] *C19MC*, which is expressed almost exclusively in placenta,[11] is believed to play important roles in the regulation of cellular differentiation, trophoblast migration and immunomodulation during pregnancy.[12, 13] Altered expression of *C19MC* has been reported in gestational hypertension, preeclampsia and fetal growth restriction.[12, 13]

Fetal growth and postnatal development, which are critical processes of life, are regulated by genetic and epigenetic factors. SNPs located within or in the vicinity of imprinted genes correlate with fetal growth characteristics in a parent-of-origin manner.[14, 15] Non-genetic variation or pathological disruption of DNA methylation marks in several imprinted loci, including *IGF2-H19*, *GNAS* or *DLK1-DIO3,* have been consistently related to changes in pre- and postnatal growth as well.[16-19]

Currently, there are no reports of parental factors related to placental *C19MC* methylation variation, nor about the impact of *C19MC* differential methylation on the offspring's development. Here, we examined for the first time the placental *C19MC* DNA methylation levels and their association with 1) parental weight and height, 2) parental transmission of haplotypes within a SNP (rs55765443) in *C19MC*, 3) postnatal growth and body composition of the offspring at birth and in childhood, and 4) gene expression levels of representative *C19MC* miRNAs.

## METHODS

*Study population and ethics*

The study population included 72 pregnant Caucasian women who delivered healthy infants, and 63 of the respective fathers who also accepted to participate in this longitudinal cohort study (Table 1). The subjects were recruited during the first trimester of pregnancy among those seen within a setting of prenatal primary care in l'Alt Empordà and Girona (North-eastern Spain) from 2008 to 2010. Information on pregnancy, labor and delivery characteristics was retrieved from standardized medical records. Pregnancies resulting from assisted reproductive technology (ART) were excluded because ART may perturb imprinting.[20] Women with major medical, surgical or obstetrical complications, including multiple pregnancies, hypertension, gestational diabetes or preeclampsia, and fetal growth restriction, malformations or asphyxia were also excluded.

The protocol was approved by the Institutional Review Board of Dr. Josep Trueta Hospital (Reference number: 2013132; Approval date: November 24th 2014) and informed written consent was obtained from all parents.

*Anthropometric assessments*

Father's weight and height were measured at inclusion without shoes and wearing light clothes; maternal weight and height were assessed similarly at each trimester of gestation. Body-mass index (BMI) was calculated as weight divided by height squared (Kg/m$^2$).

All infants were born at term of pregnancy. After delivery, weight and length were measured using a calibrated scale and a measuring board. Gestational age- and sex-adjusted z-scores for birth weight and length were calculated using regional norms.[21] From the children included at birth, those whose parents agreed that they could participate further in the study (n=32) were followed-up at the age of 6 years. Their characteristics at birth did not differ from those who did not participate in the follow-up study. Weight was measured on a calibrated scale wearing light clothes, and height was measured with a Harpenden stadiometer without shoes. BMI and age- and sex-adjusted z-scores were calculated as above. Waist circumference was measured in the supine position at the umbilical level. Hip circumference was measured at the widest part, at the level of the greater trochanters.

*Fat measurements*

Fat mass was assessed by bioelectric impedance (Hydra Bioimpedance Analyzer 4200; Xitron Technologies, San Diego CA), at the age of 6 years, as previously described in prepubertal children.[22]

Visceral fat was estimated as described by Hirooka et al[23] using high-resolution ultrasonography (MyLabTM25, Esaote, Italy) in a transverse abdominal scan with a convex 3-3.5 MHz transducer, with minimal pressure applied to prevent compression of the fat layers. Measurement of visceral fat by ultrasound correlates well with that measured by computed tomography.[23, 24] All images were obtained with the subject in supine position at the end of a normal exhalation and performed by the same observer. The average of three measurements was used for all sites; the intra-subject coefficient of variation was <6%.

*Sample collection*

Blood samples were drawn from both progenitors in EDTA tubes at the initial visit. Samples were centrifuged at 2000 x g for 15 minutes at 4ºC and total leukocytes were harvested. The placentas were collected immediately after childbirth. Three cuboidal biopsies

(1 $cm^3$) containing placental villous tissue, were dissected from the non-membranous surface (maternal side) of placentas after removing the decidua (outermost layer) midway between the umbilical cord and the placental margin. The same location was used when sampling all placentas to reduce interplacental variability. All samples were stored at -80ºC.

*DNA methylation analysis*

Quantitative DNA methylation analysis was performed by pyrosequencing of bisulphite-treated DNA.[25] Genomic DNA was extracted from placentas using the Gentra PureGene tissue kit (Qiagen, Germany). Sodium bisulfite conversion of 500 ng of DNA was performed using the EZ DNA Methylation-Gold kit (Zymo Research, Irvine CA). Bisulphite-treated DNA (20 ng) was amplified with 0.3 µM of forward and biotinylated reverse primers (Supplementary Table S1). Reactions were performed in 1X Taq GOLD buffer adjusted to 1.5 mM $MgCl_2$, 0.2 mM dNTPs and 1 U of Taq GOLD polymerase (Qiagen) in a total volume of 35 µl. PCR cycling steps were: 15 min at 94°C followed by 40 cycles of 20 s at 94°C, 30 s at 58.6ºC and 1 min at 72°C, with a final extension of 10 min at 72°C. PCR product was rendered single-stranded[25] and 4 pmol of the sequencing primer (Supplementary Table S1) were added following pyrosequencing in PyroMark Q96 ID and Q96 MD instruments (Qiagen). Raw data were analyzed using the Q-CpG software (V.1.0.9, Biotage AB), which calculates the ratio of converted Cs (Ts) to unconverted Cs at each CpG, giving the percentage of methylation. All reactions were run in duplicates. We analyzed 5 consecutive CpG dinucleotides located within the *C19MC* ICR (chr19:53648001-53648160 from UCSC Genome Browser, Human Dec. 2013 (GRCh38/hg38) Assembly, Supplementary Figure S1A). The overall DNA methylation level across the *C19MC* ICR was defined as the average of methylation at all 5 CpG sites.

*Genotyping (Restriction Fragment Length Polymorphisms, RFLPs)*

Mother-father-child trios were genotyped in order to study the paternal and maternal transmission of rs55765443 SNP alleles to the child. To assess parental genotype, DNA was extracted from parental blood leukocytes using the Gentra PureGene blood kit (Qiagen). For fetal genotyping, DNA was extracted from placentas as described above. DNA (10 ng) was amplified in a 10 µl reaction with 1x $NH_4$ buffer supplemented with 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 U BIOTAQ DNA Polymerase (Bioline, Memphis TN) and 0.6 pmol/µl of each primer. PCR primers (chr19:53665044; UCSC Genome Browser, Human Dec. 2013 (GRCh38/hg38) Assembly) were: forward 5'-TGTGGCCAGACTTTAATCCA-3' and reverse 5'-TTGGAGATTTTAGGGGGAGTC-3'. PCR conditions were: 94ºC for 5 min; 20 cycles of 94ºC 45 s, 64ºC 45 s (decreasing 0.5ºC each cycle) and 72ºC 45s; 15 cycles of 94ºC 45 s, 54ºC 45 s and 72ºC 45s; and 72ºC for 10 min. PCR product (207 bp) was then digested with 1 U of *Bso*BI restriction enzyme (New England Biolabs, Ipswich Massachusetts) at 37ºC for 16 hours. The fragments were visualized by electrophoresis through a 2% (w/v) agarose gel. The different genotypes were assessed according to the size and pattern of distribution of the fragments (Homozygous T: 1 band 207 bp; Heterozygous: 3 bands of 207, 152 and 55 bp; Homozygous G: 2 bands of 152 and 55 bp). Mother-father-child trios that were all heterozygous were uninformative for imputing parental allelic transmission, thus the final number of samples used in the analysis was 53 trios. All SNP genotypes were shown to be consistent with Hardy–Weinberg equilibrium ($\chi^2$ test p=0.900).

*Gene expression analysis by Real Time-PCR*

Total RNA was extracted and retrotranscribed using the RNeasy mini kit (Qiagen) and the Megaplex Human RT Primers (Pool A v2.1) with MultiScribe Reverse Transcriptase (Thermofisher Scientific, Waltham, MA). The following TaqMan Gene Expression assays (Thermofisher Scientific) were used to amplify the cDNA: miR-517a (Ref#002402), miR-517c (Ref#001153), miR-520c (Ref#002389), miR-520g (Ref#001121) and the endogenous controls miR-523 (Ref#002386), miR-532 (Ref#001518) and miR-425-5p0 (Ref#425-5p).[26] Reactions were run on a LightCycler 480 Real-Time PCR System (Roche Diagnostics, Rotkreuz, Switzerland), using the default cycling conditions. Relative mRNA levels were calculated according to the $2^{-\Delta CT}$ method.

*Statistics*

Statistical analyses were performed using the SPSS 22.0 package (SPSS Inc). Non-normally distributed data was log-transformed to improve symmetry. The relationship between DNA methylation levels and both anthropometric parameters and gene expression was tested by Pearson correlation followed by multiple regression analysis using the enter method to adjust for possible confounding variables (maternal pre-gestational and gestational weight, maternal height, gestational age, birth weight or length, and child's sex, age and BMI). Differences in DNA methylation levels among haplotypes were examined by unpaired Student's T-test. Accepting an alpha risk of 0.05 in a bilateral contrast, the study has an 80% power to detect a significant Pearson correlation coefficient of at least 0.5 between variables, and a difference of at least 5 units in DNA methylation between groups assuming a standard deviation (SD) of 5 units (GRANMO, IMIM, version 7.12). When analyzing the percentage of methylation of each CpG, the statistical significance was set at 0.0125 after applying the

Bonferroni correction for subgroup analyses (0.05/4 subgroup analyses: maternal characteristics, paternal characteristics, data at birth and childhood data).

# RESULTS

*Subjects*

Table 1 shows the clinical variables in the parents and their children, at birth and at 6 years of age. Maternal anthropometric variables (height, pre-gestational and predelivery weights, and pregestational BMI) associated with offspring's variables (weight, height and BMI) at birth and 6 years of age (Supplementary Table S2).

*DNA Methylation levels at C19MC in placental tissue*

DNA methylation levels of the 5 studied CpGs within the ICR region of *C19MC* (Supplementary Figure S1A) were assessed by pyrosequencing. The percentage of DNA methylation in placenta for each CpG and the average methylation for all CpGs are shown in Supplementary Figure S1B. Average methylation levels of the combined CpGs at *C19MC* ICR ranged from 26 to 56% with mean and SD values of 42.7 ± 7.9 %. The methylation levels at the different CpGs analyzed were highly concordant. This indicates that methylation was affected similarly across the entire genomic region.

*Higher maternal size correlates with lower C19MC methylation in the placenta*

Firstly, we aimed to study the relationship between methylation levels at the *C19MC* ICR and parental phenotype. Higher maternal pre-gestational weight (r=–0.316, p=0.007), height (r=–0.291, p=0.013) and BMI (r=–0.237, p=0.045), as well as pre-delivery weight (r=–0.376, p=0.002), associated with lower mean methylation levels at the placental *C19MC* ICR (Figure 1 A-C and Supplementary Table S3). Paternal anthropometric variables were not related to methylation levels within the placental *C19MC* cluster (Supplementary Table S3).

Following correction for multiple testing, maternal pre-gestational weight, height and pre-delivery weight remained significantly associated with mean methylation at *C19MC* ICR (all p≤0.01). Maternal pre-gestational weight (p=0.011), height (p=0.019) and pre-delivery weight (p=0.003) independently explained mean placental *C19MC* methylation levels in multivariate linear models after adjusting for the following confounding variables: maternal age, gestational age and child's sex, with model $R^2$ of 0.05, 0.04 and 0.10, respectively (Supplementary Table S4).

*Parentally transmitted alleles for the rs55765443 SNP and placental C19MC DNA methylation*

We further assessed whether placental *C19MC* ICR methylation levels were related to the parental transmission of the rs55765443 SNP within the *C19MC* cluster. Maternally transmitted haplotypes had no significant effect on placental *C19MC* methylation levels (Supplementary Figure S2A and Table S5).

Fetal inheritance of the paternal T allele was associated with lower methylation levels (39.8 ± 2.0 %) when compared with the G allele (47.2 ± 2.0 %) only at CpG5 of *C19MC ICR* (p=0.05, Supplementary Table S5). As for mean *C19MC* ICR methylation levels, the difference between T (41.0 ± 1.4 %) and G alleles (45.3 ± 1.6 %, Supplementary Figure 2B and Table S5) approached significance (p=0.059). However, none of these associations remained significant after correction for multiple testing.

*Lower placental C19MC methylation levels associates with bigger offspring's size at 6 years of age*

Next, we examined the consequences of the differential DNA methylation at the placental *C19MC* ICR on the offspring at birth and at age 6 years. At birth, no statistically significant relationships between newborns' anthropometric variables and *C19MC* methylation levels were observed (Supplementary Table S6). However, lower mean methylation levels at placental *C19MC* ICR correlated with larger children's size and fat abundance at age 6 years [weight ($p \leq 0.001$), height ($p=0.001$), BMI ($p=0.017$), waist ($p=0.003$), hip ($p=0.003$), fat mass ($p=0.005$) and visceral fat ($p=0.009$); Figure 2 and Supplementary Table S6].

The correlations between methylation at *C19MC* ICR and weight, height, waist circumference, hip circumference, fat mass or visceral fat in the offspring remained significant after correction for multiple testing (all $p \leq 0.0125$). Placental *C19MC* ICR methylation levels also remained independently associated with children's anthropometric variables (weight, height, waist and hip) after adjusting for confounding variables, including maternal weight or height, in general linear models (Table 2). The association of *C19MC* ICR methylation with fat mass, but not that with visceral fat, was also independent of confounding variables (Table 2).

*Placental C19MC ICR methylation levels and gene expression*

Finally, we investigated the potential link between *C19MC* ICR methylation and gene expression in the placental samples. Due to the complexity of this locus, which is transcribed as a single pri-miRNA and post-transcriptionally processed to generate numerous miRNAs with a wide range of expression levels,[27, 28] we quantified the expression of representative miRNAs (miR-517a, miR-517c, miR-520c and miR-520g) in 56 placental samples. These miRNAs were chosen in order to have diverse expression levels (high, medium, low) and a wide coverage across the cluster. Median expression of the 4 analyzed miRNAs was used in order to approximate the gene expression levels of *C19MC*. In a bivariate correlation analysis, increased *C19MC* ICR methylation associated with reduced median miRNA expression (r=

–0.267, p=0.047; Figure 3). This finding suggests that ICR methylation negatively affects miRNA expression across the locus.

## DISCUSSION

Although altered expression of imprinted gene clusters has been linked to postnatal growth disorders,[29] evidence that the placental imprinted *C19MC* cluster may play a role in this process is missing. Here, we show for the first time that, in the placenta, the level of methylation at the *C19MC* ICR is linked to maternal weight and height, and is ultimately related to the offspring's body size and body composition in childhood.

Interestingly, placental *C19MC* ICR methylation associated with the offspring's anthropometry, independently of maternal weight and height. The association found in our cohort, between maternal weight/height and offspring's weight/height, may be explained at least partially by placental *C19MC* ICR methylation levels. Thus, besides genetic factors,[30] the offspring's size may be significantly modulated by epigenetic factors at early stages of life. More generally, and in agreement with our finding, gene variants associated with height or BMI have been found to account for only around 20% of the heritable phenotypic variation.[31, 32]

Different studies have shown that maternal size correlates with the offspring's growth and body composition.[33-35] Our results suggest a possible role for placental *C19MC* ICR methylation in the modulation of childhood growth. Similarly, placental DNA methylation at the *IGF2-H19*, *GNAS* and *DLK1-DIO3* imprinted loci has been linked to pre and postnatal growth characteristics.[16, 18, 19] Moreover, our results link placental *C19MC* ICR methylation to the body composition of the offspring in childhood. Interestingly, maternal obesity has been suggested to alter adipocyte commitment and differentiation in the offspring via an epigenetic mechanism as well.[36] Our findings suggest that maternal size and offspring fat mass at 6 years of age may, at least in part, be linked to *C19MC* methylation.

It has been shown that maternal factors, such as nutrient supplementation or alcohol intake, can alter DNA methylation patterns of imprinted genes with paternal expression, for instance at *MEST*, *PLAGL1* or *IGF2*.[37-40] It is therefore plausible that maternal body weight or height, may also influence placental methylation as well. In principle, the maternal allele is virtually 100% methylated at *C19MC*, as opposed to the paternal allele, which is 0% methylated. However, we found *C19MC* ICR global methylation to be less than 50% in most cases. This suggested that the maternal allele had partially lost its imprinting marks, thus allowing limited maternal expression and probably leading to functional consequences due to the high dosage sensitivity of imprinted gene expression.[41]

In contrast to pre-gestational maternal weight, gestational weight gain cannot be directly linked to placental *C19MC* ICR methylation. Indeed, previous studies have suggested that nutrition throughout gestation does not contribute to the epigenetic reprogramming of the ICRs of *GNAS*, *GRB10*, *KCNQ1OT1/CDKN1C* and *H19/IGF2* loci.[42, 43] These findings could be related to the imprinted condition of the studied gene clusters. DNA methylation at ICRs of imprinted genes is acquired during gametogenesis, thus establishing germline-derived differentially methylated regions, and is refractory to the genome-wide methylation reprogramming that occurs in the embryo after fertilization.[20, 29, 44] Accordingly, maternal weight during oogenesis and early embryogenesis, rather than gestational weight gain, could affect the DNA methylation at the imprinted *C19MC* ICR.

DNA methylation levels can also be modified by the presence of specific SNPs.[45] SNPs neighboring CpG islands, such as rs55765443, can lead to allele-specific DNA methylation changes, usually in a cis-acting mechanism.[46] In this line, several obesity-associated SNPs have been reported to be associated with methylation levels at proximal CpG sites.[47] In the *C19MC* cluster, the maternal allele is methylated by a specific imprint acquired in oocytes,[7] thus it would be plausible that a SNP in the maternally transmitted allele could affect *C19MC* ICR methylation levels. We therefore studied the relationship of the maternal and paternal alleles for the rs55765443 SNP with methylation levels. While no association was evident for transmission of the maternal allele and *C19MC* ICR methylation levels, we found a tendency for the paternally transmitted allele to be associated with altered methylation levels, which was not confirmed in adjusted analyses. We cannot rule out the possibility that other SNPs located close to this region could be linked to altered methylation levels of the *C19MC* ICR.

Epigenetic variations at placental *C19MC* may be associated with concordant levels of change in gene expression that could explain the observed link between the cluster and offspring's anthropometry. In fact, *C19MC* microRNAs can traffic among placental, maternal and fetal compartments,[48] feasibly influencing fetal programming and thus the developmental trajectory. Computational and bioinformatic analyses have predicted that *C19MC* miRNAs participate in reproduction, development, and differentiation,[49] including stem cell self-renewal and pluripotency by controlling G1-S transition and apoptosis signaling.[50] Indeed, members of the *C19MC* cluster have been shown to regulate cellular reprogramming, enhance proliferation and suppress apoptosis.[51] Regarding our results, a possible hypothesis is that differential methylation at the *C19MC* cluster may cause molecular changes in fetal tissues, probably in stem cells, through variations in microRNAs levels. *C19MC* microRNAs may thus target and prime specific fetal tissues, such as adipose tissue, for enhanced proliferation later in life. This hypothesis could not be tested in our study. Instead, we analyzed the expression levels of representative *C19MC* miRNAs in placental tissue and found that methylation levels in this locus associated with miRNA expression levels. Although the molecular changes may be rather fast, the anthropometric changes (weight, height or fat mass) could need more time to be revealed, and thus they may not be seen until childhood, explaining the lack of association with infants' anthropometry.

We acknowledge some study limitations. The specific role for the rs55765443 paternal haplotype on *C19MC* methylation, as well as the interaction between the paternally transmitted allele and maternal size as modifiers of the placental *C19MC* ICR methylation levels, need to be addressed in additional studies. A next step should also include the analysis in fetal tissues of gene expression levels of the 56 microRNAs encoded by *C19MC* cluster, which was beyond the scope of the current study. Finally, other factors such as diet or other environmental factors were not included in the current analysis but should be considered for further studies as possible confounders.

The strengths of our study include the follow-up data on the offspring. Moreover, due to the crucial role of imprinted genes in early life development, the significance of our study is warranted, since elucidating the factors that affect their epigenetic regulation has important implications for understanding the causes of human health and disease, and may help to establish protocols for early detection/prevention of adult diseases. Finally, the use of placenta to predict complications later in life would have obvious advantages, as it is an easily available tissue that can be sampled non-invasively.

In summary, maternal size associates with the percentage of methylation within the placental *C19MC* ICR, and such methylation levels are related to offspring's size and body composition at age 6 years. Increased maternal size may reduce placental *C19MC* methylation, in turn leading to larger size of the offspring in childhood. These results may help to establish protocols for early detection/prevention of childhood/adulthood diseases related to body size and composition.

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The authors declare that they have no conflicts of interest.

Supplementary information is available at International Journal of Obesity's website.

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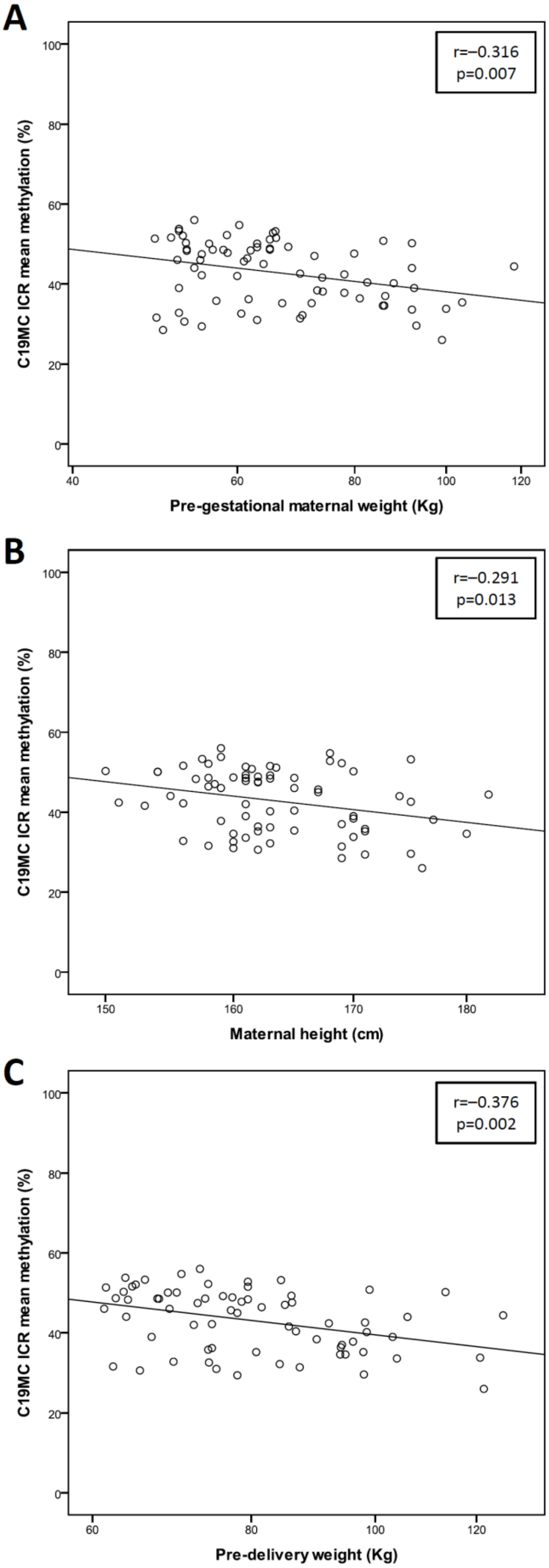
**FIGURE LEGENDS**

**Figure 1. Higher maternal size correlated with lower *C19MC* methylation in placenta. (A)** Scatter plot of pre-gestational maternal weight and *C19MC* ICR mean methylation. **(B)** Scatter plot of maternal height and *C19MC* ICR mean methylation. **(C)** Scatter plot of pre-delivery maternal weight and *C19MC* ICR mean methylation. Pearson correlation statistics are shown within each box.

**Figure 2. Lower placental *C19MC* ICR methylation associated with greater offspring's size and adiposity at 6 years of age.** Scatter plots showing the correlation of placental *C19MC* ICR methylation levels with offspring's weight z-score **(A)**, height z-score **(B)**, waist circumference **(C)**, hip circumference **(D)**, fat mass **(E)** and visceral fat volume **(F)** at 6 years of age. Pearson correlation statistics are shown in the boxes.

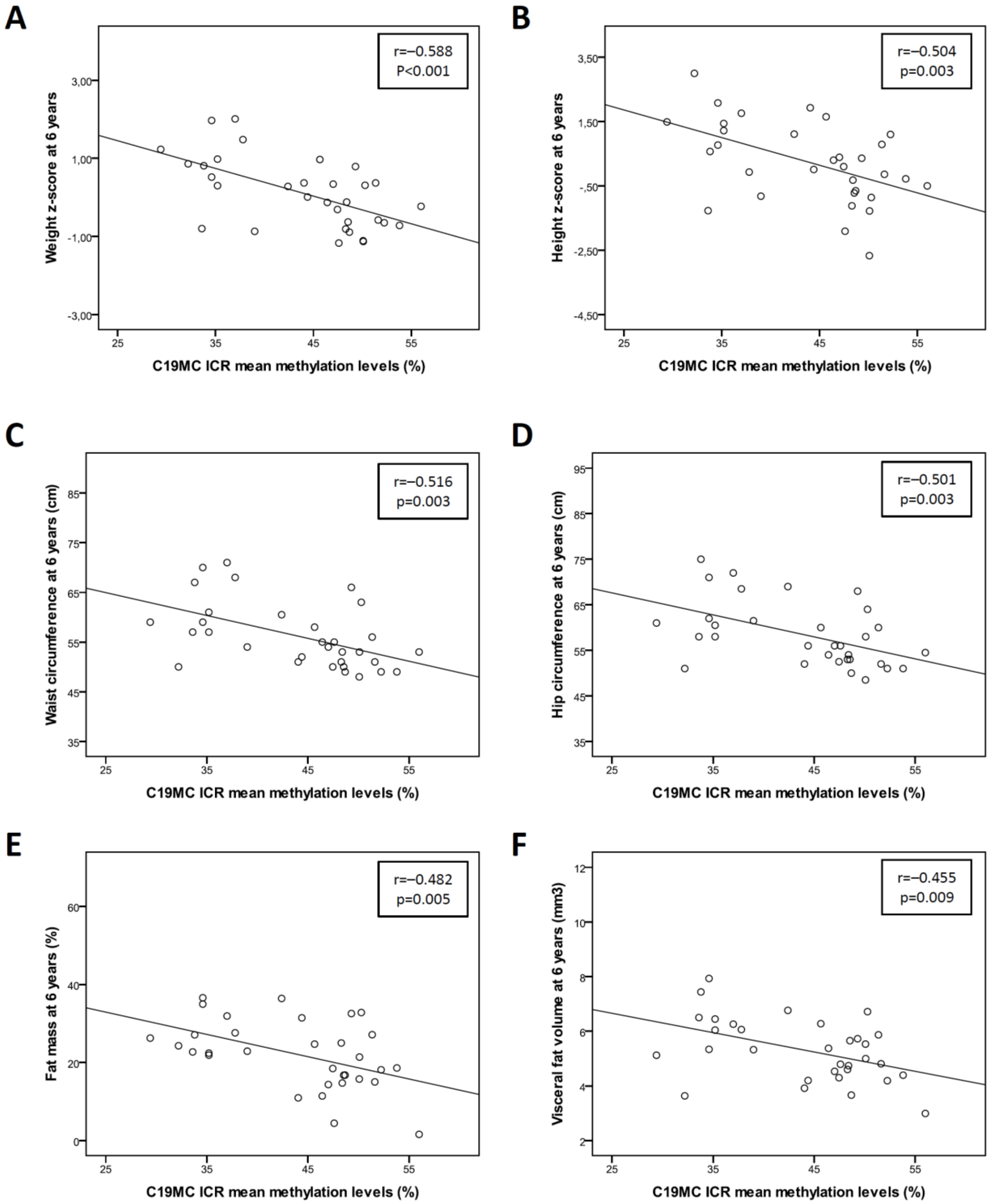
**Figure 3. Placental methylation levels at the C19MC ICR and gene expression.** Scatter plot showing the correlation of placental *C19MC* ICR methylation levels with median *C19MC* gene expression (n=56). Pearson correlation statistics are shown in the box.

# Figure 1


A
r=−0.316
p=0.007
C19MC ICR mean methylation (%)
0
20
40
60
80
100
40
60
80
100
120
Pre-gestational maternal weight (Kg)
B
r=−0.291
p=0.013
C19MC ICR mean methylation (%)
0
20
40
60
80
100
150
160
170
180
Maternal height (cm)
C
r=−0.376
p=0.002
C19MC ICR mean methylation (%)
0
20
40
60
80
100
60
80
100
120
Pre-delivery weight (Kg)

1

# Figure 2


A
r=−0.588
P<0.001
Weight z-score at 6 years
C19MC ICR mean methylation levels (%)
B
r=−0.504
p=0.003
Height z-score at 6 years
C19MC ICR mean methylation levels (%)
C
r=−0.516
p=0.003
Waist circumference at 6 years (cm)
C19MC ICR mean methylation levels (%)
D
r=−0.501
p=0.003
Hip circumference at 6 years (cm)
C19MC ICR mean methylation levels (%)
E
r=−0.482
p=0.005
Fat mass at 6 years (%)
C19MC ICR mean methylation levels (%)
F
r=−0.455
p=0.009
Visceral fat volume at 6 years (mm3)
C19MC ICR mean methylation levels (%)

# Figure 3

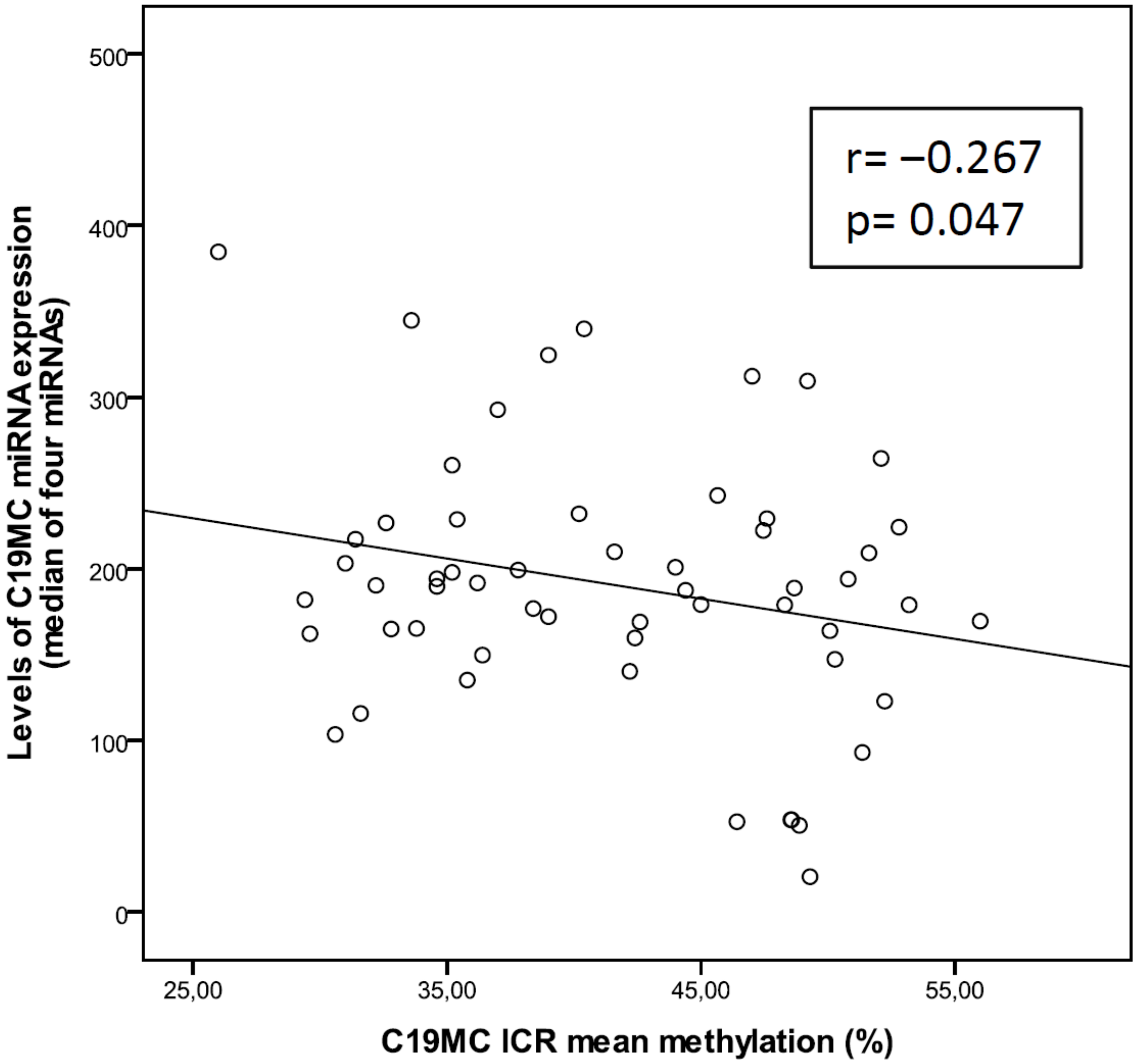

r= −0.267
p= 0.047
Levels of C19MC miRNA expression
(median of four miRNAs)
500
400
300
200
100
0
25,00
35,00
45,00
55,00
C19MC ICR mean methylation (%)

**Table 1.** Clinical assessments in the studied subjects.

| | | |
|---|---|---|
| **Mothers** | N | 72 |
| | Age at conception (yr) | 30.7 ± 3.9 |
| | Primiparous (%) | 58.6 |
| | Pre-gestational weight (Kg) | 63.5 (55.0-78.0) |
| | Height (cm) | 162 (159-169) |
| | Pre-gestational BMI (kg/m$^2$) | 23.6 (21.4-30.3) |
| | Gestational weight gain (kg) | 13.2 (10.8-17.2) |
| | Pre-delivery weight (kg) | 78.0 (69.1-93.4) |
| **Fathers** | N | 63 |
| | Weight (Kg) | 80.0 (75.0-86.2) |
| | Height (cm) | 176 ± 6 |
| | BMI (kg/m$^2$) | 26.0 (24.2-27.2) |
| **Newborns** | N | 72 |
| | Sex (% female) | 51.4 |
| | Gestational age (wk) | 40 ± 1 |
| | Weight (g) | 3304 ± 367 |
| | Weight z-score | 0.1 ± 0.9 |
| | Length (cm) | 49.4 ± 1.8 |
| | Length z-score | -0.3 ± 1.0 |
| **Follow-up at age 6 years** | N | 32 |
| | Age (yr) | 5.8 ± 0.8 |
| | Sex (% female) | 58 |
| | Weight (kg) | 22.0 ± 3.8 |
| | Weight z-score | 0.1 ± 0.9 |
| | Height (cm) | 116 ± 6 |
| | Height z-score | 0.2 ± 1.3 |
| | BMI (kg/m$^2$) | 16.2 (15.1-17.2) |
| | BMI z-score | 0.0 ± 0.8 |
| | Waist circumference (cm) | 56.2 ± 6.6 |
| | Hip circumference (cm) | 58.5 ± 7.1 |
| | Fat mass (%) | 22.1 ± 8.8 |
| | Visceral fat volume (mm$^3$) | 5.3 ± 1.2 |

BMI: body-mass index.
Data are expressed as mean ± SD or median (interquartile range) for Gaussian and non-Gaussian variables, respectively.

**Table 2.** Six independent multivariate linear models of placental *C19MC ICR* methylation levels and children's characteristics at follow-up (age of 6, n=32).

| | β | p | Total $R^2$ |
|---|---|---|---|
| **Weight (kg)** | | | **0.44** |
| *C19MC ICR mean methylation (%)* | -0.552 | 0.003 | |
| *Age(y)* | 0.325 | 0.039 | |
| Non-explaining variables: sex, birth weight z-score, pre-gestational maternal weight. | | | |
| **Height (cm)** | | | **0.39** |
| *C19MC ICR mean methylation (%)* | -0.486 | 0.009 | |
| *Age (Y)* | 0.361 | 0.022 | |
| Non-explaining variables: sex, birth length z-score, maternal height. | | | |
| **Waist circumference (cm)** | | | **0.33** |
| *C19MC ICR mean methylation (%)* | -0.497 | 0.003 | |
| Non-explaining variables: sex, age. | | | |
| **Hip circumference (cm)** | | | **0.41** |
| *C19MC ICR mean methylation (%)* | -0.449 | 0.004 | |
| *Age (y)* | 0.412 | 0.007 | |
| Non-explaining variables: sex. | | | |
| **Fat mass (%)** | | | **0.56** |
| *C19MC ICR mean methylation (%)* | -0.428 | 0.004 | |
| *Sex* | 0.502 | <0.0001 | |
| Non-explaining variables: age, BMI (Body-mass index). | | | |
| **Visceral fat ($mm^3$)** | | | **0.72** |
| *Age (y)* | 0.523 | <0.0001 | |
| *BMI ($kg/m^2$)* | 0.456 | <0.0001 | |
| Non-explaining variables: sex, *C19MC* ICR mean methylation. | | | |

In bold: dependent variable for each multivariate linear model.

# SUPPLEMENTAL MATERIAL

## TABLES

**Table S1.** Primer sequences for bisulfite PCR and pyrosequencing of *C19MC* ICR, corresponding to chr19:53648001-53648160 (hg38).

| | **Sequence** | **Product length (bp)** |
|---|---|---|
| **Forward primer** | 5'-TGTTTGGAAAGGGGTTGTTTATGTA-3' | 160 |
| **Reverse primer** | 5'-**Biotin**-CCCTCAAAAAAAAACCAAAATATTAATTC-3' | |
| **Sequencing primer** | 5'-GTTTTGGATAGAGGTTTTTAGAG-3' | |

**Table S2**. Pearson correlation coefficients between maternal and offspring's anthropometry.

| | Offspring's variables | | | | |
|---|---|---|---|---|---|
| | **Birth Height SDS (n=72)** | **Height SDS at 6 years (n=32)** | **Birth Weight SDS (n=72)** | **Weight SDS at 6 years (n=32)** | **BMI SDS at 6 years (n=32)** |
| **Maternal variables** | | | | | |
| Height | 0.257* | 0.316* | ns | ns | ns |
| Pregestational weight | ns | ns | 0.286* | 0.329* | ns |
| Predelivery weight | ns | ns | 0.350** | 0.450** | ns |
| Pregestational BMI | ns | ns | ns | ns | 0.318* |

BMI: body-mass index; SDS: standard deviation score; ns: non-significant.
*p≤0.05; **p≤0.01

**Table S3**. Pearson correlation coefficients between parental anthropometry, the percentage of methylation at placental *C19MC* ICR for each studied CpG and the mean overall methylation.

| | ***C19MC* ICR methylation levels (%)** | | | | | |
|---|---|---|---|---|---|---|
| | **CpG1** | **CpG2** | **CpG3** | **CpG4** | **CpG5** | **Mean of all 5 CpGs** |
| **Mothers' parameters (n=72)** | | | | | | |
| Pre-gestational weight | -0.280* | **-0.312**** | **-0.286**** | -0.276* | **-0.336**** | **-0.316**** |
| Height | **-0.286**** | -0.223 | 0.279* | -0.237* | **-0.338**** | **-0.291**** |
| Pre-gestational BMI | -0.197 | -0.261* | -0.207 | -0.214 | -0.239* | -0.237* |
| Gestational weight gain | 0.014 | 0.027 | -0.039 | -0.011 | -0.184 | -0.052 |
| Pre-delivery weight | **-0.324**** | **-0.332**** | **-0.338**** | **-0.315**** | **-0.441***** | **-0.376**** |
| **Fathers' parameters (n=63)** | | | | | | |
| Weight | -0.059 | -0.012 | -0.038 | -0.061 | -0.015 | -0.025 |
| Height | -0.060 | 0.011 | -0.109 | -0.112 | 0.002 | -0.054 |
| BMI | -0.040 | -0.011 | -0.010 | -0.016 | -0.018 | -0.002 |

BMI: body-mass index
*p≤0.05; **p≤0.01; ***p≤0.001
Correlations that remained significant after multiple testing correction (p≤0,01; see text) are highlighted in bold.

**Table S4.** Three independent multivariate linear models of placental *C19MC* ICR methylation levels (n=72) and maternal characteristics.

| | β | p | $R^2$ |
|---|---|---|---|
| ***C19MC* ICR mean methylation (%)** | | | |
| Pre-gestational weight (kg) | -0.307 | 0.011 | 0.05 |

Non-explaining variables: maternal age, gestational age and child's sex.

| | β | P | $R^2$ |
|---|---|---|---|
| ***C19MC* ICR mean methylation (%)** | | | |
| Height (cm) | -0.294 | 0.019 | 0.04 |

Non-explaining variables: maternal age, gestational age and child's sex.

| | β | P | $R^2$ |
|---|---|---|---|
| ***C19MC* ICR mean methylation (%)** | | | |
| Pre-delivery weight (Kg) | -0.371 | 0.003 | 0.10 |

Non-explaining variables: maternal age, gestational age and child's sex.

In bold: dependent variables in each multivariate linear model.

**Table S5.** Percentage of *C19MC* ICR methylation levels and the corresponding parentally transmitted alleles (n=53).

| | ***C19MC* ICR methylation levels (%)** | | | | | | |
|---|---|---|---|---|---|---|---|
| | **n** | **CpG1** | **CpG2** | **CpG3** | **CpG4** | **CpG5** | **Mean of all 5 CpGs** |
| **Maternally transmitted allele** | | | | | | | |
| T | 44 | 41.8 ± 1.1 | 42.1 ± 1.2 | 44.2 ± 1.2 | 42.2 ± 1.2 | 42.5 ± 1.8 | 42.6 ± 1.2 |
| G | 9 | 44.7 ± 3.2 | 45.7 ± 2.6 | 46.9 ± 2.7 | 46.4 ± 2.7 | 46.4 ± 3.0 | 46.0 ± 2.8 |
| **Paternally transmitted allele** | | | | | | | |
| T | 32 | 40.6 ± 1.3 | 40.5 ± 1.4 | 43.1 ± 1.5 | 41.1 ± 1.4 | 39.8 ± 2.0* | 41.0 ± 1.4 |
| G | 21 | 43.9 ± 1.6 | 44.6 ± 1.7 | 46.1 ± 1.6 | 44.7 ± 1.7 | 47.2 ± 2.0 | 45.3 ± 1.6 |

Results are mean ± SEM.*p≤0.05 for an unpaired Student's T-test.

**Table S6**. Pearson correlation coefficients between children's variables and the percentage of methylation at placental *C19MC* ICR for each studied CpG and the overall methylation.

| | ***C19MC* ICR methylation levels (%)** | | | | | |
|---|---|---|---|---|---|---|
| | **CpG1** | **CpG2** | **CpG3** | **CpG4** | **CpG5** | **Mean of all 5 CpGs** |
| **Children's parameters at birth (n=72)** | | | | | | |
| Placental weight (g) | -0.035 | 0.009 | -0.041 | -0.032 | -0.086 | -0.042 |
| Weight (g) | -0.103 | -0.085 | -0.140 | -0.147 | -0.179 | -0.141 |
| Weight z-score | -0.005 | -0.022 | -0.101 | -0.082 | -0.144 | -0.081 |
| Length (cm) | -0.127 | -0.095 | -0.122 | -0.186 | -0.177 | -0.152 |
| Length z-score | -0.046 | -0.034 | -0.084 | -0.130 | -0.150 | -0.098 |
| **Children's parameters at age 6 years (n=32)** | | | | | | |
| Weight (kg) | **-0.546*****| **-0.636***** | **-0.547***** | **-0.575***** | **-0.629*****| **-0.607***** |
| Weight z-score | **-0.543***** | **-0.567***** | **-0.548***** | **-0.588***** | **-0.591***** | **-0.588**** |
| Height (cm) | **-0.569***** | **-0.578***** | **-0.518**** | **-0.592***** | **-0.522**** | **-0.573***** |
| Height z-score | **-0.531**** | **-0.444**** | **-0.483**** | **-0.581***** | **-0.412*** | **-0.504**** |
| BMI ($kg/m^2$) | -0.325 | **-0.451**** | -0.379* | -0.352* | **-0.508**** | -0.420* |
| BMI z-score | -0.282 | -0.402* | -0.338 | -0.312 | -0.412* | -0.377* |
| Waist circumference (cm) | **-0.438**** | **-0.568***** | **-0.466**** | **-0.448**** | **-0.568***** | **-0.516***** |
| Hip circumference (cm) | **-0.427**** | **-0.559***** | **-0.456**** | **-0.445**** | **-0.532**** | **-0.501**** |
| Fat mass (%) | **-0.458**** | **-0.505**** | **-0.505**** | -0.386* | **-0.459**** | **-0.482**** |
| Visceral fat volume ($mm^3$) | -0.396* | **-0.524**** | **-0.431**** | -0.377* | **-0.472**** | **-0.455**** |

BMI: body-mass index
*p≤0.05; **p≤0.01; ***p≤0.001. Highlighted in bold are those correlations that remained significant after multiple testing correction (p<0,0125; see text).

**FIGURES**

**Figure S1. *C19MC* locus and methylation levels. (A)** Localization of the *C19MC* ICR (Imprinting Control Region). The region analyzed for methylation studies was chr19:53648001-53648160, and the SNP (rs55765443) localization was chr19:53665044 according to the GRCh38/hg38 assembly. **(B)** *C19MC* ICR methylation levels in placenta. Boxplots show the percentage of DNA methylation in *C19MC* ICR (boxes represent median ± interquartile range) for each CpG and average of percent methylation for all studied CpGs within the *C19MC* ICR.

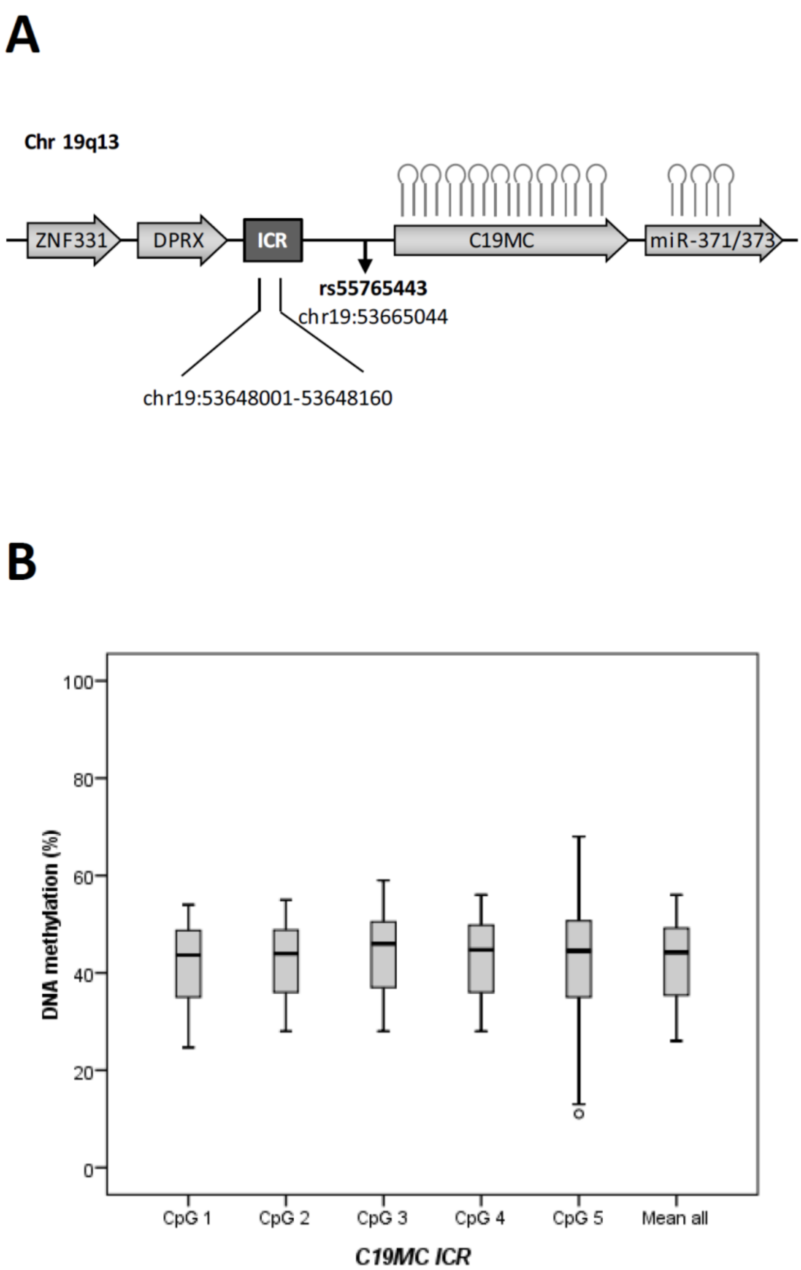



**Figure S2.**

**Parentally transmitted alleles for the rs55765443 SNP and placental *C19MC* ICR methylation. (A)** Mean methylation levels at *C19MC* ICR according to the maternally transmitted allele (p=0.271). **(B)** Mean methylation levels at *C19MC* ICR according to the paternally transmitted allele (p=0.059).