

# Beckwith–Wiedemann syndrome prenatal diagnosis by methylation analysis in chorionic villi

Leda Paganini<sup>1</sup>, Nicole Carlessi<sup>1</sup>, Laura Fontana<sup>1</sup>, Rosamaria Silipigni<sup>2</sup>, Silvia Motta<sup>2</sup>, Stefano Fiori<sup>1</sup>, Silvana Gueneri<sup>2</sup>, Faustina Lalatta<sup>3</sup>, Anna Cereda<sup>4</sup>, Silvia Sirchia<sup>5</sup>, Monica Miozzo<sup>1,\*</sup>, and Silvia Tabano<sup>1</sup>

<sup>1</sup>Division of Pathology; Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico; Department of Pathophysiology & Transplantation; Università degli Studi di Milano; Milano, Italy; <sup>2</sup>Medical Genetics Laboratory; Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico; Milano, Italy; <sup>3</sup>Clinical Genetics Unit; Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico; Milano, Italy; <sup>4</sup>Pediatric Unit; Azienda Ospedaliera Papa Giovanni XXIII; Bergamo, Italy; <sup>5</sup>Medical Genetics; Department of Health Sciences; Università degli Studi di Milano; Milano, Italy

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**Abbreviations:** ART, Assisted Reproductive Technologies; AS, Angelman Syndrome; BWS, Beckwith–Wiedemann Syndrome; CV, Chorionic Villi; CVC, Cultured Chorionic Villi; CVF, Fresh Chorionic Villi; DMR, Differentially Methylated Regions; ICR, Imprinting Control Region; IUGR, Intra-Uterine Growth Restriction; MMD, Multilocus Methylation Defect; PWS, Prader–Willi Syndrome; SGA, Small for Gestational Age; SRS, Silver Russell Syndrome; UPD, Uniparental Disomy; US, Ultra Sound.

Beckwith–Wiedemann syndrome (BWS) is an imprinting disorder that can be prenatally suspected or diagnosed based on established clinical guidelines. Molecular confirmation is commonly performed on amniocytes. The possibility to use fresh (CVF) and cultured (CVC) chorionic villi has never been investigated. To verify whether CVF and CVC are reliable sources of DNA to study fetal methylation, we used pyrosequencing to test the methylation level of a number of differentially methylated regions (DMRs) at several imprinted loci (ICR1, ICR2, *H19*, *PWS/AS-ICR*, *GNASXL*, *GNAS1A*, *ZAC/PLAGL1*, and *MEST*) and at non-imprinted *MGMT* and *RASSF1A* promoters. We analyzed these regions in 19 healthy pregnancies and highlighted stable methylation levels between CVF and CVC at ICR1, ICR2, *GNASXL*, *PWS/AS-ICR*, and *MEST*. Conversely, the methylation levels at *H19* promoter, *GNAS1A* and *ZAC/PLAGL1* were different in CVC compared to fresh CV. We also investigated ICR1 and ICR2 methylation level of CVF/CVC of 2 BWS-suspected fetuses (P1 and P2). P1 showed ICR2 hypomethylation, P2 showed normal methylation at both ICR1 and ICR2. Our findings, although limited to one case of BWS fetus with an imprinting defect, can suggest that ICR1 and ICR2, but not *H19*, could be reliable targets for prenatal BWS diagnosis by methylation test in CVF and CVC. In addition, *PWS/AS-ICR*, *GNASXL*, and *MEST*, but not *GNAS1A* and *ZAC/PLAGL1*, are steadily hemimethylated in CV from healthy pregnancies, independently from culture. Thus, prenatal investigation of genomic imprinting in CV needs to be validated in a locus-specific manner.

## Introduction

Genomic imprinting is a mammalian epigenetic phenomenon that results in monoallelic gene expression determined by the parental origin of the allele.<sup>1</sup> Almost all imprinted genes are organized in clusters and have differences in their CpG site-methylation profile at key regulatory elements.<sup>2</sup>

The role of DNA methylation in gene expression is well characterized at the imprinted locus 11p15. This cluster comprises 2 distinct domains regulated by the imprinting control regions 1 (ICR1) and 2 (ICR2).<sup>3</sup> Genetic and epigenetic defects at this cluster are associated with Beckwith–Wiedemann syndrome (BWS, OMIM: #130650) and Silver–Russell syndrome (SRS, OMIM: #180860), which are growth disorders with opposite

phenotypes. In healthy individuals, the paternally derived ICR1 allele is methylated and the maternal one is unmethylated; at ICR2, the opposite methylation pattern is observed. BWS may arise from one of the following mechanisms: hypomethylation at ICR2 (50–60% of cases); hypermethylation at ICR1 (2–8% of cases); mosaic segmental paternal uniparental disomy (UPD) (10–20% of cases); and mutations of the maternal *CDKN1C* allele (5–10% of cases).<sup>4</sup> Conversely, SRS is associated with hypomethylation of ICR1 (35–50% of cases), duplication of the maternal 11p15.5 (unknown frequency), maternal UPD of chromosome 7 (about 10% of the cases), and deletions/duplications of the same chromosome (rare).<sup>5</sup>

In the last few years, several groups<sup>6–8</sup> have demonstrated that DNA methylation defects in imprinting disorders can involve

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\*Correspondence to: Monica Miozzo; Email: monica.miozzo@unimi.it

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multilocus methylation defects (MMDs) of imprinted loci. For example, Azzi et al. first demonstrated that 9.5% of SRS and 24% of BWS have MMDs at additional DMRs other than 11p15.5.<sup>8</sup> No significant differences in clinical features exist between BWS patients with multilocus or single-locus methylation defect.<sup>8</sup>

BWS can be prenatally suspected, or provisionally diagnosed, applying the guidelines proposed by Williams et al.<sup>9</sup> They set up a diagnostic scheme based on the identification of at least 2 major findings in ultrasound (US) examination (i.e., an abdominal wall defect, macroglossia, or macrosomia), or one major and 2 minor findings (i.e., nephromegaly/dysgenesis, adrenal cytomegaly, aneuploidy/abnormal loci, or polyhydramnios).<sup>9</sup> Molecular confirmation of BWS is essential for an accurate prenatal counseling that considers the possibility of recurrence and the postnatal risk of additional BWS manifestations such as childhood cancers. BWS patients have increased risk of developing certain embryonic tumors: Wilms' tumor, adrenocortical carcinoma, hepatoblastoma, gonadoblastoma, rhabdomyosarcoma, and neuroblastoma.<sup>9</sup> In particular, 28.6% and 17.3% of the patients with hypermethylation at ICR1 and 11p15 UPD, respectively, develop Wilms' tumors during infancy. By contrast, the prevalence of childhood cancers in patients with hypomethylated ICR2 or *CDKN1C* mutations is 3.1% and 8.8%, respectively.<sup>10,11</sup> The risk of BWS recurrence in the same family depends on the genetic/epigenetic defect in the proband: the risk is low in absence of genomic rearrangements (deletions/duplications) and in the presence of single or multilocus methylation defects and UPD. On the contrary, the recurrence risk may be as high as 50% in those with genomic defects or *CDKN1C* mutations. These data support the need for an early and accurate molecular diagnosis of BWS to the tailored surveillance of patients.

In contrast to BWS, SRS diagnosis is mainly performed after birth in infants with growth restriction, hypoglycemia, body-asymmetry, triangular facial appearance, and clinodactyly.<sup>12</sup> During prenatal life, the main US manifestation is intrauterine growth restriction, which is an unspecific condition. Therefore, prenatal molecular investigation is rarely prompted only by fetal morphology.

Beside the presence of morphological fetal defects, molecular prenatal testing for imprinting diseases can be prompted by the finding of fetal/placental karyotypes that may predispose to UPD formation (i.e., Robertsonian and reciprocal translocations, structural rearrangements, and confined placental mosaicism involving chromosomes with imprinted genes).

Moreover, imprinting disorders are possibly associated with assisted reproductive technologies (ART);<sup>13,14</sup> concerning BWS, it seems to be more frequent in ART than in spontaneous conceptions.<sup>15</sup>

For prenatal methylation analyses, both chorionic villi (CV) and amniocytes are eligible DNA sources.<sup>11,16</sup> CV sampling offers the advantage of an earlier diagnosis than amniocentesis with comparable risks,<sup>17</sup> even if it should be considered that the methylation of the placenta is generally lower than that of the embryo.<sup>16,18</sup> Furthermore, when an imprinting disease is

suspected after CV karyotyping, the left over cultured villi could represent a unique and easily available source of fetal DNA for methylation analysis during the first trimester of pregnancy. For this reason, it is important to ascertain whether villi culturing influences the methylation pattern.

The aim of our study was to assess the feasibility of prenatal diagnosis (PD) of methylation defects, in particular BWS, in fresh (CVF) and cultured CV (CVC). We analyzed normal and pathological pregnancies to investigate CV methylation patterns at both imprinted and non-imprinted regions, as well as the effect of culturing on methylation profile. In particular, we evaluated ICR1, ICR2, the *H19* promoter (11p15.5), PWS/AS-ICR (15q11–q13), *GNASXL* and *GNASIA* (20q13.32), *ZAC/PLAG1* (6q24) and *MEST* (7q32) imprinted loci, and the promoters of the non-imprinted *MGMT* and *RASSF1* genes.

## Results

### CV do not modify the methylation pattern at ICR1 and ICR2 during culturing

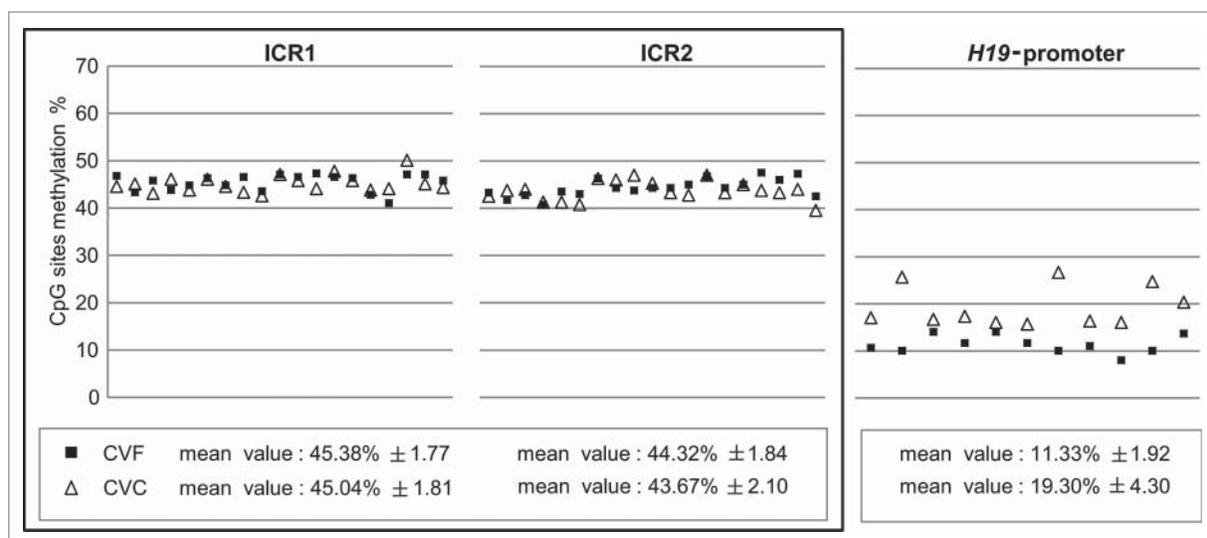
Since BWS is mainly linked to imprinting defects at chromosome cluster 11p15.5, we first investigated the methylation status at ICR1, ICR2, and *H19* promoter in 19 normal pregnancies. Mean ICR1 and ICR2 methylation percentages were  $45.38 \pm 1.77\%$  and  $44.32 \pm 1.84\%$  in CVF, respectively, and  $45.04 \pm 1.81\%$  and  $43.67 \pm 2.10\%$  in CVC, respectively (Fig. 1). These results demonstrate that CVF and CVC methylation levels at these imprinted control regions are very similar ( $P = 0.457$  and  $P = 0.176$  for ICR1 and ICR2, respectively), indicating that the culturing of CV does not modify ICR1 and ICR2 physiological methylation pattern.

In contrast, we found that the *H19* promoter was hypomethylated in both CVF ( $11.33 \pm 1.92\%$ ) and CVC ( $19.30 \pm 4.30\%$ ), compared to the expected profile for an imprinted locus. This result is consistent with *H19* promoter methylation pattern in third trimester healthy pregnancies, previously identified by our group<sup>16</sup>. In addition, the *H19* mean methylation levels in CVC were higher than those in CVF ( $P = 0.0005$ ) (Fig. 1), indicating an effect of CV culture on *H19* promoter methylation.

### Methylation analysis in fresh and cultured CV requires gene-specific validation

Given the results obtained at 11p15.5 imprinted DMRs, to ascertain the feasibility of methylation analyses at other imprinted loci, we extended our study to 5 additional imprinted loci: *GNASXL* and *GNASIA* (20q13.32), PWS/AS-ICR (15q11–q13), *ZAC/PLAG1* (6q24) and *MEST* (7q32) (Fig. 2). As control genes, we studied the methylation profile of non-imprinted *MGMT* and *RASSF1* gene promoters.

We found that *GNASXL*, PWS/AS-ICR, and *MEST* were stably hemimethylated in both CVF (*GNASXL*:  $39.16\% \pm 1.98$ , PWS/AS-ICR:  $43.70\% \pm 4.60$ , *MEST*:  $51.40\% \pm 3.11$ ) and CVC (*GNASXL*:  $39.88\% \pm 1.97$ , PWS/AS-ICR:  $43.15\% \pm 3.41$ , *MEST*:  $50.74\% \pm 4.20$ ) (Fig. 2A). These results highlight that, at these DMRs, CV methylation



**Figure 1.** Quantitative CpG methylation analysis of ICR1, ICR2 and *H19* in CVF and CVC from normal pregnancies. Symbols represent mean methylation percentage of at least two independent experiments for each sample. Black full squares symbolize fresh chorionic villi (CVF); empty triangles cultured chorionic villi (CVC). Mean percentage value is calculated on a different number of CpG sites analyzed at each locus. White boxes report mean methylation level  $\pm$  SD calculated for all the analyzed CVF and CVC. Loci that maintain stable methylation values (ICR1 and ICR2) in CVF and CVC are grouped together in a full-line square.

pattern is not influenced by cell culturing (*GNASXL*:  $P = 0.48$ ; *PWS/AS-ICR*:  $P = 0.766$ , *MEST*:  $P = 0.73$ ). In addition, the methylation values at all these DMRs were as expected for imprinted loci and similar to those found in peripheral lymphocytes from healthy donors (data not shown).

On the contrary, *GNAS1A* imprinted gene was severely hypomethylated in CVF and CVC (CVF:  $22.85\% \pm 4.08$ ; CVC:  $8.95\% \pm 3.17$ ) and exhibited methylation instability after culture ( $P = 2.44486E-06$ ) (Fig. 2B). Similarly, *ZAC/PLAG1* methylation was not stable after culture ( $P = 0.0016$ ) but showed CVF and CVC methylation values in accordance with an imprinted locus (CVF:  $40.69\% \pm 1.12$ , CVC:  $45.30\% \pm 3.15$ ) (Fig. 2B). *GNAS1A* and *ZAC/PLAG1* were hemimethylated in peripheral lymphocytes from healthy donors (data not shown).

Finally, concerning the non-imprinted investigated loci, *MGMT* promoter was unmethylated in both CVF and CVC (CVF:  $2.06 \pm 0.48\%$ , CVC:  $2.16 \pm 0.53\%$ ), without statistically significant differences before and after CV culture ( $P = 0.605$ ) (Fig. 2C). Methylation values of the exon 1 CpG sites of the *RASSF1* gene were  $52.50 \pm 7.33\%$  in CVF and  $28.00 \pm 11.10\%$  in CVC (Fig. 2C), indicating that the methylation levels at this locus were consistently different after culture ( $P = 3.37 \times 10^{-7}$ ).

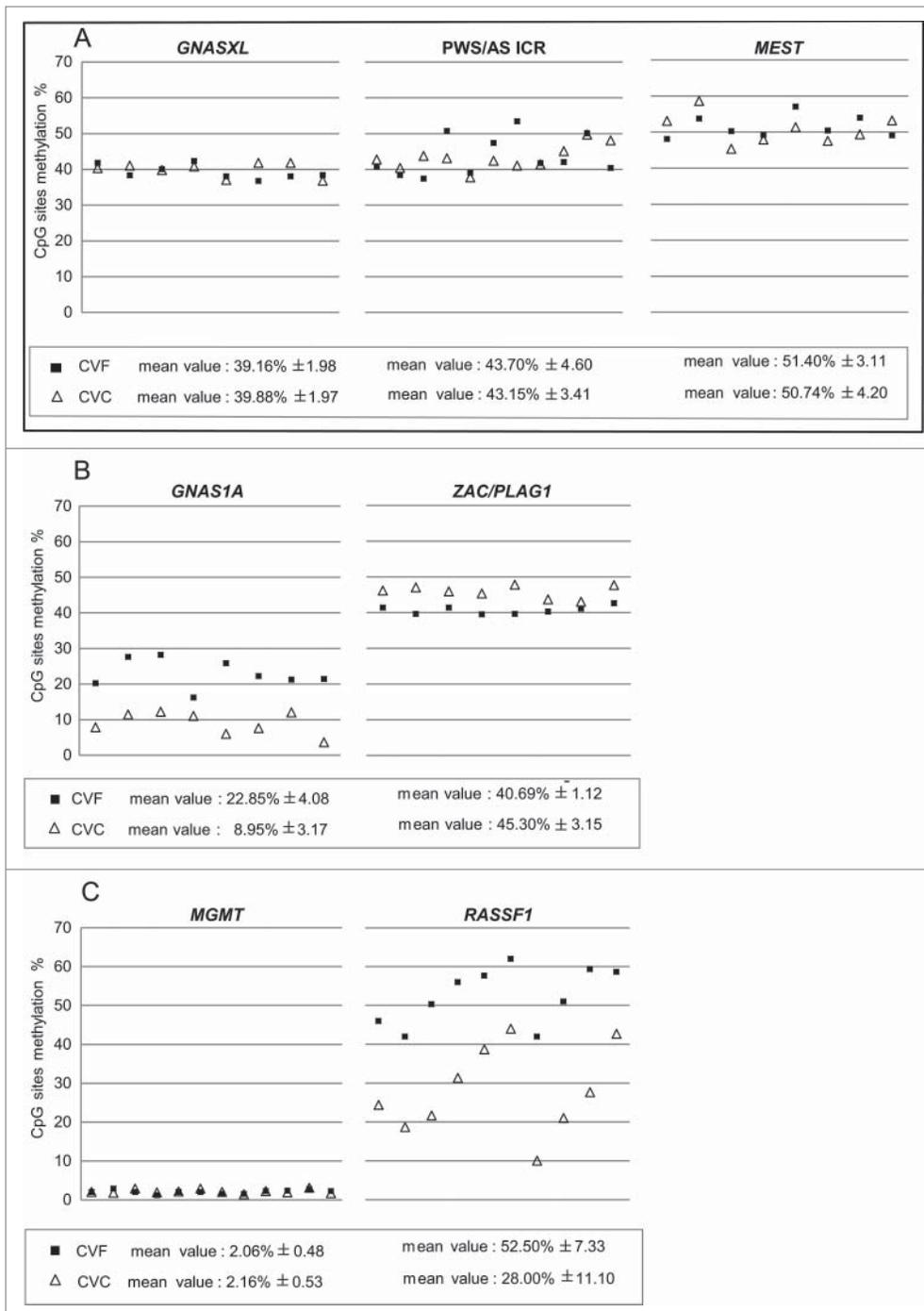
#### Prenatal diagnosis of suspected BWS can be performed in fresh as well as in cultured CV

Given that the CV methylation levels of ICR1 and ICR2 were consistent with those expected for imprinted loci and stably maintained throughout culture in healthy pregnancies, we analyzed these regions in CVF and CVC from 2 fetuses (P1 and P2) with US anomalies suggestive for BWS.

At 11p15.5 imprinted region P1 had normal methylation at ICR1 and hypomethylation at ICR2 in both CVF (ICR1:  $48.92 \pm 2.13\%$ ; ICR2:  $17.63 \pm 0.88\%$ ) and CVC (ICR1:  $45.25 \pm 2.63\%$ ; ICR2:  $16.13 \pm 0.18\%$ ) (Fig. 3A and C). Autopsy confirmed the presence of omphalocele. Hematoxylin/eosin staining of the placenta highlighted stem villi hyperplasia, focal vesicle-like villi and abnormal vascularization, thus supporting the US evidence for mesenchymal dysplasia (Fig. 3B). ICR2 hypomethylation was also present in all the fetal specimens obtained from P1 autopsy, including skin ( $15.5 \pm 1.00\%$ ), inner intestine ( $11.0 \pm 1.41\%$ ), kidney ( $15.75 \pm 0.96\%$ ), and intestine protruding from omphalocele ( $8.75 \pm 1.71\%$ ); of note, the latter sample had the lowest methylation value (Fig. 3C).

Since in P1 the molecular results confirmed the clinical diagnosis of BWS, to explore the possibility of MMDs, we carried out methylation analysis in CVF and CVC of imprinted loci (*GNASXL*, *PWS/AS-ICR* and *MEST*) that showed stable methylation in CV. We observed normal methylation values at all the investigated loci in both CVF (*GNASXL*:  $45.00\% \pm 4.89$ ; *PWS/AS-ICR*:  $46.33\% \pm 1.15$ ; *MEST*:  $46.50\% \pm 5.00$ ) and in CVC (*GNASXL*:  $42.75\% \pm 3.40$ ; *PWS/AS-ICR*:  $47.50\% \pm 2.08$  and *MEST*:  $48.75\% \pm 2.63$ ) (*GNASXL*:  $P = 0.53$ ; *PWS/AS-ICR*:  $P = 0.43$ ; *MEST*:  $P = 0.45$ ). These methylation values were also confirmed in fetal autaptic tissues (Fig. 3C), allowing us to exclude MMDs.

P2 displayed normal methylation levels at ICR1 and ICR2 in both CVF (ICR1:  $46.38 \pm 1.59\%$ ; ICR2:  $47.88 \pm 0.53\%$ ) and CVC (ICR1:  $46.00 \pm 4.32\%$ ; ICR2:  $49.00 \pm 3.77\%$ ) (Fig. 3A). Since P2 was negative for 11p15.5 methylation defects, as second step of the diagnostic flowchart, *CDKN1C* sequencing was performed. We found a heterozygous missense mutation, *c.491C>T* (*p.Pro164Leu*), located within exon 1 encoding for the PAPA domain of the CDKN1C protein. This variant, not



**Figure 2.** Quantitative CpG methylation analysis of imprinted and non-imprinted loci in CVF and CVC from normal pregnancies. The following loci were analyzed: (A) *GNASXL*, *PWS/AS-ICR*, *MEST*; (B) *GNAS1A*, *ZAC/PLAG1*; (C) *MGMT* and *RASSF1*. Symbols represent mean methylation percentage of at least two independent experiments for each sample. Black full squares symbolize fresh chorionic villi (CVF); empty triangles cultured chorionic villi (CVC). Mean percentage value is calculated on a different number of CpG sites analyzed at each locus. White boxes report mean methylation level  $\pm$  SD calculated for all the analyzed CVF and CVC. Loci that maintain stable methylation values (*GNASXL*, *PWS/AS-ICR*, *MEST*) in CVF and CVC are grouped together in a full-line square.

BWS, starting from the methylation analysis at 11p15.5, is usually performed on amniocytes. However, molecular tests on CV could be performed earlier in the pregnancy, and CV could be cultured to provide DNA for additional molecular investigations.

In normal pregnancies, we explored by pyrosequencing the feasibility of methylation analysis of CVF and CVC at different imprinted (*ICR1*, *ICR2*, *H19* promoter, *GNASXL*, *GNAS1A*, *ZAC/PLAG1* and *MEST*) and non-imprinted (*MGMT* and *RASSF1*) loci. We also investigated 2 fetuses in which BWS was suspected because of the presence of omphalocele.

We found a high degree of stability in the methylation levels

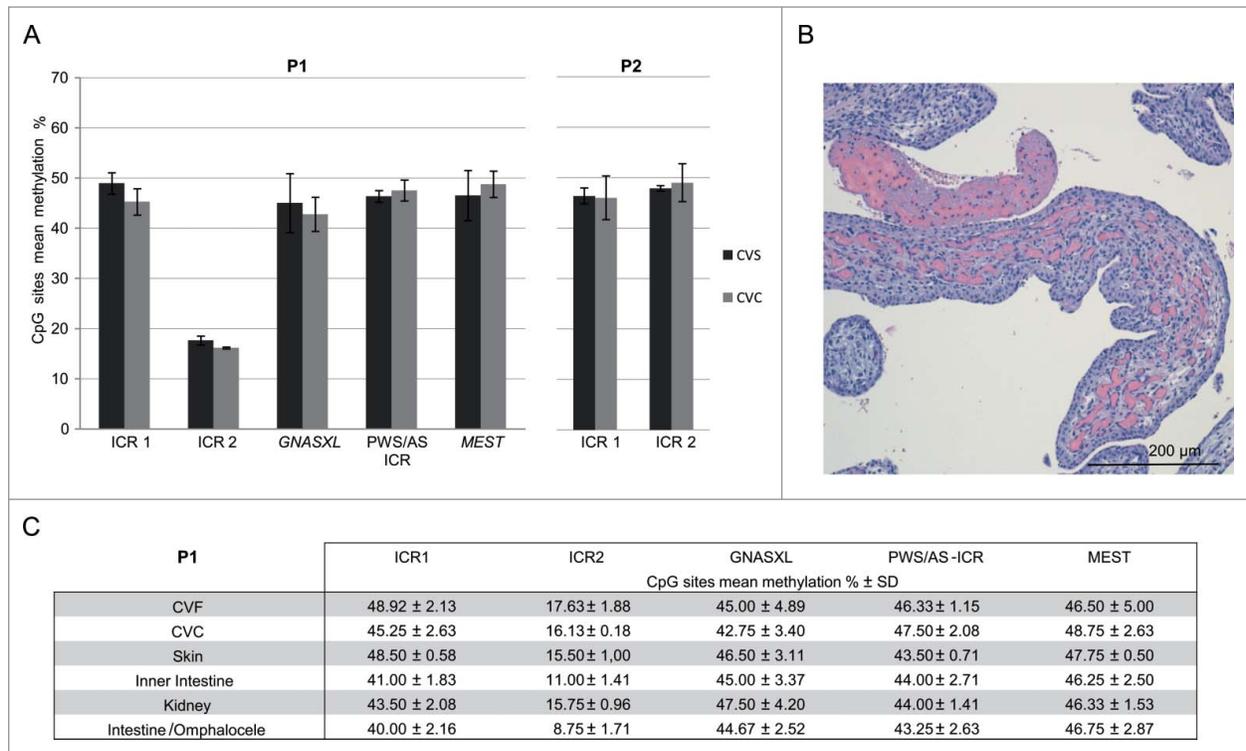
previously described, was inherited from the mother and confirmed in the fetus (data not shown). PolyPhen 2 ([www.genetics.bwh.harvard.edu/pph2/](http://www.genetics.bwh.harvard.edu/pph2/)) functional prediction suggested that it was benign.

## Discussion

Prenatal molecular diagnosis of imprinting disorders is crucial in presence of fetal anomalies, particular fetal chromosomal abnormalities or a positive family history. The prenatal test for

between CVF and CVC at *ICR1*, *ICR2*, *GNASXL*, *PWS/AS-ICR* and *MEST*. Furthermore, these regions were imprinted in CV, indicating that they did not undergo the genome-wide wave of demethylation characteristic of the placenta. We therefore surmise that these DMRs can be reliable targets for PD using CV.

Conversely, the imprinted locus *H19*, despite its chromosome position at 11p15.5 cluster, was hypomethylated and instable after CV culturing, in agreement with our previous observations.<sup>16</sup> The same trend was observed for *GNAS1A* imprinted gene that was hypomethylated in both CVF and CVC, with CVC showing a significant reduction of methylation. *ZAC/*



**Figure 3.** BWS-suspected pregnancies (P1 and P2) molecular results. **(A)** Histograms of the quantitative CpG methylation analysis performed at ICR1, ICR2, *GNASXL*, *PWS/AS-ICR* and *MEST* imprinted loci on P1 fetus and at ICR1 and ICR2 on P2 BWS-suspected case. Black bars represent CVS samples; gray bars CVC samples. **(B)** Hematoxylin/eosin staining of P1 placenta showing hypercellularity (fibroblasts overgrowth), enlargement of stem villi, hypervascularized villi (chorangiosis) with focal dilatation, branching, and sinusoid formation (H&E, 10x). **(C)** Table reporting P1 mean methylation percentage  $\pm$  SD at ICR1, ICR2, *GNASXL*, *PWS/AS-ICR* and *MEST* in CVF, CVC, skin, inner intestine, kidney and intestine protruding from omphalocele.

*PLAG1* imprinted locus was hemimethylated in CVF, as expected, but this pattern was not maintained after culture, where it was observed a slight, but significant, increased methylation. Thus, our data suggest that the methylation analysis of *H19*, *GNASIA* and *ZAC/PLAG1* promoters is not reliable for PD.

We also evaluated the effect of culturing on the methylation of the non-imprinted genes *MGMT* and *RASSF1*. *MGMT* is a housekeeping gene,<sup>19</sup> therefore its promoter is unmethylated. This pattern was present in CVF and maintained also after culture. Conversely, *RASSF1* methylation is not maintained in CVC. Chiu et al.<sup>20</sup> analyzed the *RASSF1* methylation profile in CVF by a semi-quantitative approach and found it to be hypermethylated compared to fetal tissues. Here, we confirmed this observation in CVF. After cell culture, the methylation consistently decreased by about 20% in each sample. This could be ascribed to culture conditions or to cell composition. Indeed, CVF samples are mainly composed of 2 different cell types: cytotrophoblasts and mesenchymal (stromal) cells. During culturing, only the mesenchymal cells expand; thus the methylation level can change if the mesenchymal cells have a different profile.

Taken together, our experiments in normal pregnancies highlight that some imprinted loci can be hypomethylated in fresh CV and that the methylation levels can vary after culturing in a locus-specific manner. Among the investigated regions,

methylation at the DMRs ICR1, ICR2, *GNASXL*, *PWS/AS-ICR* and *MEST* seems not to be influenced by cell culture.

Our conclusions are strengthened by the methylation analyses of the BWS case P1, in which we found ICR2 hypomethylation in CVF as well as in CVC and in the tissues analyzed after abortion. In this case we also tested *GNASXL*, *PWS/AS-ICR* and *MEST* DMRs and excluded the MMD condition. Although in this case it was possible to perform PD for BWS using fresh and cultured CV, our observations essentially derived from one affected fetus only. In addition, since several imprinting disorders are mosaic, it is feasible that the placental and fetal tissues may have different methylation profile. Therefore, additional cases are needed to better define the possibility to detect methylation defects in placenta of suspected BWS fetuses.

P1, like the majority of BWS individuals conceived after ART, had methylation defects.<sup>21</sup> The severity of the hypomethylation at the affected locus and the persistence of the methylation anomaly in multiple anatomic sites suggest that the defect was on the maternally inherited 11p15 allele and already present in the oocyte. This methylation defect could be a consequence of the ovarian stimulation during ART procedures. Finally, P1 also presented with placental mesenchymal dysplasia, which is reported in 19% of BWS cases.<sup>22</sup> This clinical feature is related, in more than half of cases, to other

pregnancy complications, including intrauterine growth restriction and/or fetal death, preterm delivery, and spontaneous preterm labor.<sup>22</sup>

P2 did not have primary ICR1/ICR2 methylation defects in either CVF or CVC. *CDKN1C* sequencing revealed a maternally inherited missense variant within exon 1 that has not been previously described and might be associated with BWS.

In summary, we showed that prenatal investigation of BWS on fresh and cultured cells of first trimester CV can be reliable because the methylation profile at ICR1 and ICR2 is maintained in the placenta and unaffected by culture. Despite this, the methylation analysis of imprinted genes in fresh and in cultured CV requires caution because the imprinting signatures in the placenta can be different from the fetal compartment and can vary after culturing in a locus-specific manner.

## Patients and Methods

### Population

Samples included in the study were 19 first-trimester CV from singleton pregnancies without prenatal signs of imprinting disorders, recruited at the Obstetric Unit of Policlinico Mangiagalli Hospital (Milan, Italy) from 2013 to 2014, after an informed consent was obtained. In 18 cases the indication for PD was the advanced maternal age; in one case it was a positive Bi-Test, a first trimester non-invasive screening to identify pregnancies at risk for fetal aneuploidies.<sup>23</sup> Mean gestational age at CV sampling was 11 weeks gestation (wg). Eighteen conceptions were spontaneous; one was from *in vitro* fertilization (IVF). The karyotype analyses did not indicate chromosomal anomalies. We analyzed both CVF and CVC from the residual biological material used for the cytogenetics. CVC were obtained after about 21 days of culture, using standard procedures and conditions.<sup>24</sup>

In addition, we investigated CVF and CVC from two pregnancies (P1 and P2) in which the suspicion of BWS arose from US investigation. P1 (12<sup>th</sup> wg) presented with both mesenchymal dysplasia and omphalocele. The pregnancy was voluntarily aborted at 14<sup>th</sup> wg, and skin, kidney, inner intestine, and intestine protruding from the omphalocele were also analyzed for their methylation pattern. Conception was obtained by IVF.

P2 (12<sup>th</sup> wg), from the Pediatric Unit of Papa Giovanni XXIII Hospital (Bergamo, Italy), had a giant isolated omphalocele, with liver and stomach herniation. Pregnancy was voluntarily terminated at 18<sup>th</sup> wg. Conception was spontaneous.

### DNA methylation analysis

Genomic DNA from CVF, CVC, and fetal fragments was isolated using QIAamp DNA Mini Kit (Qiagen) and quantified by the NanoDrop ND1000 spectrophotometer (NanoDrop Technologies). Sodium bisulfite conversion of DNA (500 ng) was performed using the EZ DNA Methylation-Gold Kit (Zymo Research Corporation). PCRs were carried out using 20 ng of

bisulfite-converted DNA and 10 pmol of forward and reverse primers, one of them biotinylated. Quantitative DNA methylation analyses were performed using a Pyro Mark ID instrument (Biotage) in the PSQ HS 96 System (Biotage), with the Pyro-Gold SQA reagent kit (Biotage). Raw data were analyzed using the Q-CpG software v1.0.9 (Biotage), which calculates the ratio of converted to unconverted cytosines at each CpG, giving the percentage of methylation. Each sample was run in duplicate, and the methylation value was considered reliable when the SD between the two independent experiments was below 5%. The given methylation value is the result of the mean methylation levels of different CpG sites at each locus. Results are shown as the mean of the analyzed CpG sites  $\pm$  SD. The methylation levels were compared using the Student's *t*-test.

We investigated the methylation of the following imprinted and non-imprinted loci (**Supplemental file 1**): ICR1, ICR2, and the *H19* promoter, located at the imprinted region 11p15.5<sup>11,16</sup>, PWS/AS-ICR, located at the imprinted region 15q11-q13<sup>25</sup>; the promoter regions of *MGMT* at 10q26; *RASSF1A* gene, located at the non-imprinted locus 3p21.3; the DMRs *GNASXL* and *GNASIA* at 20q13.32, *MEST* at 7q32.2 and *ZAC/PLAG1* at 6q24.2. Genomic positions, primer sequences, PCR and pyrosequencing conditions are detailed in **Supplemental file 1**.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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### Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

### Authors' Contributions

MM, ST, and SS: conception and design of the study and writing of the manuscript. LP, NC, LF: laboratory experiments, data interpretation, and writing of the manuscript. RS, SM, SF, SG, FL and AC: materials providing and data interpretation. All authors read and approved the manuscript.

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