Clinical Epigenetics

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Highly variable genomic methylation in the Beckwith-Wiedemann syndrome associated with multi-locus imprinting disturbances

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Abstract

Background The expression of imprinted genes, which depends on their gamete of origin, is regulated by DNA sequences characterized by differential methylation between the maternal and paternal alleles (also known as germline differentially methylated regions or gDMRs). The most common molecular defect associated with Beckwith-Wiedemann syndrome (BWS), a condition linked to overgrowth and tumours, is the loss of methylation of the *KCNQ1OT1*-TSS gDMR located on chromosome 11p15.5 (also known as IC2 LoM). Approximately one-third of BWS patients with IC2 LoM exhibit multi-locus imprinting disturbances (MLID). While maternal-effect variants in proteins of the oocyte subcortical maternal complex (SCMC) have been linked to MLID, the underlying mechanisms and health impact of this epigenetic disturbance remain unclear.

Results We used the Infinium EPIC methylation array to investigate whole-genome CpG methylation in 64 BWS patients with IC2 LoM and 37 control subjects. We distinguished two patient groups, one with a variable methylation level of 24 gDMRs and the other with single-locus IC2 LoM. We observed that the mothers of the former patient group carried more variants in maternal-effect genes than those of the latter group, and 50% of them, but none of the latter group had variants in the SCMC genes. Additionally, in the former group, the mothers were older at the time of pregnancy, and the patients showed higher variation in methylation levels of thousands of CpGs located in non-imprinted loci, including protochaderins and cancer-associated genes. We found no differences in clinical features or in the incidence of assisted reproductive technology between the two patient groups. However, multiple affected siblings and recurrent miscarriages were observed only among cases with biallelic maternal-effect SCMC gene variants.

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Conclusions This study demonstrates that the BWS patients with MLID exhibit highly variable methylation changes that affect both imprinted and non-imprinted loci in a seemingly stochastic manner throughout the genome. These findings support the hypothesis that MLID results from the interaction of maternal-effect genes and environmental factors in aged oocytes, leading to disordered DNA methylation in the whole genome. Future research should investigate whether and how these epimutations impact the health of affected individuals, particularly in adulthood.

Keywords DNA methylation, Genomic imprinting, Maternal-effect genes, Subcortical maternal complex, Imprinting disorders

Background

In its canonical form, genomic imprinting is a regulatory mechanism by which genes are preferentially expressed from one of the two parental alleles based on differential DNA methylation [1]. Imprinted genes are generally organized in clusters, each controlled by an Imprinting Centre (IC), a CpG-rich region characterized by germline-derived differential DNA methylation (gDMR). Further DMRs (somatic or secondary DMRs, sDMRs) that depend on hierarchical interactions with the gDMRs are established in the imprinted gene clusters during development [2]. Imprinting is required for normal development, and in humans its dysregulation leads to diseases affecting growth, metabolism and neurobehaviour collectively known as Imprinting Disorders (ImpDis) [3]. ImpDis are typically characterized by dysregulation of the imprinted genes of a cluster that is often associated with hypo- or hyper-methylation of a single gDMR. The imprinted DMRs (iDMRs) commonly associated with ImpDis are called clinical DMRs, the others non-clinical DMRs [4]. In many ImpDis cases the methylation abnormality is not limited to one gene cluster, but affects multiple iDMRs located in different chromosome regions (a condition called multi-locus imprinting disturbance, MLID) [5].

The Beckwith-Wiedemann Syndrome (BWS, OMIM: 130,650) is an ImpDis characterized by the presence of macroglossia, abdominal wall defects and lateralized overgrowth as well as other less frequent clinical features including predisposition to several paediatric tumours [6]. The most common molecular defects of BWS affect the expression of the imprinted gene cluster located on chromosome 11p15.5. This cluster is organized in two functional domains: the telomeric domain including the *IGF2* and *H19* genes that is controlled by the *H19/IGF2*:IG-DMR, and the centromeric domain including *CDKN1C* and *KCNQ1OT1* that is controlled by the *KCNQ1OT1*:TSS-DMR. The most frequent molecular abnormalities of BWS are: loss of methylation at *KCNQ1OT1*:TSS-DMR (also known and here reported as IC2 LoM, 50% of cases), uniparental paternal disomy of chromosome 11p15.5 (upd(11)pat, 20% of cases), gain of methylation at *H19/IGF2*:IG-DMR (5–10% of cases), loss of function variants of *CDKN1C* (5% of cases) and copy number variants (CNVs) of chromosome 11p15.5 (1–2% of cases).

Approximately one-third of the BWS patients with IC2 LoM display mosaic MLID [4]. The mechanism by which MLID arises in these patients remains a matter of investigation. However, recent studies have involved maternal-effect variants in the aetiology of MLID. In particular, variants in genes encoding the subcortical maternal complex (SCMC) members were described in healthy women with offspring affected by BWS and other ImpDis with MLID [5]. The SCMC is a multi-protein complex that stores proteins in oocytes that are needed for development and epigenetic reprogramming of the pre-implantation embryo [7, 8]. The clinical impact of MLID is still undefined, but increased recurrence risk and association with reproductive problems have been reported in cases with SCMC gene variants [4, 9].

ImpDis diagnosis is usually obtained with the Methylation-Specific Multiple Ligation-Dependent Probe Amplification (MS-MLPA) assay that targets a restricted number of imprinted loci and CpGs [3]. A few studies recently investigated a broader number of imprinted DMRs by employing the NGS-based ImprintSeq technique or the Infinium methylation array [10–12]. Both methods analyse approximately 50 iDMRs [13] identified previously through a combination of whole-genome bisulphite sequencing and high-density methylation microarrays [14, 15]. Among these, 12 iDMRs are located within imprinted loci associated with ImpDis, and these are typically assessed using MS-MLPA, which remains the most widely employed molecular diagnostic tool in clinical practice. However, DNA methylation outside of the imprinted loci has not been generally analysed so far. Thus, the impact of ImpDis on the whole epigenome is still unknown.

Here, we report a study on 64 BWS patients with IC2 LoM, in which the whole epigenome of the patients and the genome of their mothers were investigated by Infinium EPIC methylation array and exome sequencing analyses, respectively. We demonstrated that all the gDMRs and a consistent fraction of the rest of the genome were affected by highly variable CpG methylation changes in a

subgroup of patients. Maternal gene variants segregated with higher CpG methylation variability in the probands, and maternal age was higher, suggesting that genetic and environmental factors interact in the oocyte to cause apparently stochastic methylation changes in the whole genome of the progeny in these cases.

Patients

The study cohort consisted of 64 BWS patients from 61 families and included five patients deriving from twin birth, two pairs of siblings, and a pair of discordant monozygotic twins. The less affected monozygotic monochorionic twin (P28 in Table S1) was included in the analysis despite a clinical score below 4 because she exhibited two signs linked to BWSp, one part of the diagnostic criteria (glabellar nevus simplex) and one not typically scored (hemangioma). From a molecular perspective, excluding P28 would not significantly impact our findings: (i) methylation analysis would remain unaffected since the twins share similar blood-based epigenomes; (ii) exome sequencing, performed on the mothers, would be unchanged; and (iii) the conclusion on absence of significant phenotypic difference between cluster 2 and cluster 3 patients is confirmed (see Results section). The clinical features and clinical scores of each patient are reported in Table S1. The patients' ages ranged from one month to 39 years. In total, 36 patients were female and 28 were male. As detailed in Table S1, 17 families, of which 16 including patients with MLID, were reported in previous publications.

Written informed consent was obtained from all patients or their parents for performing and publishing the results of the molecular analyses. The research work was carried out in accordance with the ethical principles and the Italian legislation. The study was approved by the Ethical Committee of the University of Campania "Luigi Vanvitelli" (Naples, Italy; approval number: 10423, 5 May 2020).

Materials and methods

DNA extraction

Genomic DNA of patients and their parents were isolated from peripheral blood leukocyte (PBL) by standard salting-out procedure [16]. DNA concentration was determined using the NanoDrop™ 2000c Spectrophotometer (Thermo Fisher Scientific).

Methylation analysis

Molecular diagnosis of BWS with IC2 LoM was originally obtained in all patients by using CoBRA or pyrosequencing preceded by bisulphite treatment, as previously reported [17]. We performed methylation array analysis on bisulphite-converted PBL DNA of 64 patients and 37 healthy individuals using the Illumina Infinium MethylationEPIC BeadChip 850 k v.1 array (Biodiversa, Italy). We analyzsd and imported the "idat" files using the "champ.load" module of the "ChAMP" R package (v.2.23.1) [18]. To normalize and remove batch effects, we applied the "champ.norm" function with the "BMIQ" parameter and the "champ.runCombat" function to correct for slide technical effect. Additionally, SNPs that could potentially impact the analysis were filtered out. This step was integrated into our main methylation array pipeline, which employs a dedicated function to load the data and exclude SNPs. The SNP list used for filtering was generated from https://zwdzwd.github.io/InfiniumAnnotation, a resource specifically tailored for 450 K and EPIC methylation arrays that accounts for population differences. After normalization and batch correction, we downloaded the imprinted region coordinates from the Human Imprinting Encyclopaedia (http://www.humanimprints.net/) and intersected them with the array probes. Considering the average value for each imprinted region, we compared the imprinted methylation levels of the patients with those of 37 unaffected controls. Values differing by ± 3 standard deviations and at least 10% from the mean of the controls were considered abnormal methylation changes. Moreover, using a regression-based approach with limma (v3.50.3) [19] and missMethyl R packages (v.1.28.0) [20], we identified both Differentially Methylated Positions (DMPs, at least 10% mean defect to the control mean and adjusted p value < 0.001) and Differentially Variable Positions (DVPs, adjusted p value < 0.05) between clusters C2 and C1 and between clusters C3 and C1. Before performing any regression analysis, we assessed the contribution of each covariate to the overall variability in the dataset. In our case, the SVD plot showed that the primary source of variability (PC1) was strongly and significantly associated with the Sample_Group (BWS vs Control). This indicates that the main differences between samples have a biological meaning. In contrast, technical factors such as Batch, Array, or Slide did not show significant contributions until PC5. Similarly, Gender and Age showed minor effects only from PC5 or PC6, respectively (Fig. S1). For this reason, we chose not to include them as covariates in the regression model. Their contribution to the overall variability is minimal, and including them would not improve the model, but on the other hand, excluding them allows a better fitting in the identification of DMPs and DVPs by avoiding unnecessary adjustment for weak or non-informative variables. After annotation to genes, we used gprofiler2 package (v.0.2.2) [21] to plot the enriched KEGG pathways using the genes covered by the EPIC array probes manifest as custom background. To infer the CNV status, we started from the rgSet and

preprocessed the data through the "preprocessIllumina" function from the minfi package (v.1.40.0) [22]. We then plotted the CNVs using the "CNV.detailplot" function from the conumee2.0 package (v2.0) [23] using the default parameters, allowing a CNV minimum size of 50 kb with at least 15 probes.

Methylation-Specific Multiple Ligation-Dependent Probe Amplification (MS-MLPA) was carried out according to the manufacturer's instructions using the SALSA MS-MLPA Kit ME034-B1 (MRC-Holland, Amsterdam, The Netherlands), as previously described [12, 24]. The amplified products were separated by capillary electrophoresis, employing an ABI 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Data were analysed using the Coffalyser software (MRC-Holland, Amsterdam, The Netherlands). The iDMR methylation levels of 13 patients were already reported [25–28].

DNA sequencing and gene variant identification

Whole-exome sequencing (WES) and in silico prediction of variant pathogenicity were carried out as previously reported [25–28]. Briefly, reads were aligned to the human genome reference assembly (GRCh37). PCR duplicates were filtered out, and the GATK v3.7 suite was used to locally realign around inferred Insertion/Deletions (InDels) and recalibrate base quality scores. Single-nucleotide variants and InDels were called using GATK HaplotypeCaller and GenotypeGVCFs and recalibrated with VariantRecalibrator. Recalibrated variants were annotated using wANNOVA. Variants with low coverage (< 15) or low quality (< 20) were filtered out. Filters on the frequency in the general population (MAF $\leqslant$ 0.1) were based on gnomAD database. We selected variants predicted as deleterious or probably deleterious by Polyphen2 or SIFT. WES and in silico prediction of variant pathogenicity were performed on 35 mothers, whose genomic DNA was available. The maternal-effect gene (MEGs) and SCMC variants identified by WES were validated by Sanger sequencing. The WES results obtained on 10 mothers of patients with MLID were reported in previous publications [25–28]. An Excel file including all genetic variants with VAF < = 10% of the 35 patient mothers analysed is available upon request.

Results

Identification of MLID by epic methylation array analysis

We determined the mean methylation level of all the iDMRs covered by the Illumina Infinium methylation EPIC array in a cohort of 64 BWS patients with IC2 LoM and 37 control individuals (Table S2). To increase specificity and avoid false positive results, we filtered out the regions covered by fewer than four probes (GPR1-AS, IGF2R:Int2, WDR27:Int13, SVOPL:alt-TSS, FANCC:Int1, IGF2:alt-TSS, MEG3/DLK1:IG, MEG8:Int2, GPR1-AS:TSS, WRB:alt-TSS, SNRPN:Int1-DMR1 and ZNF597:3) and all the sDMRs not associated with ImpDis. Then, we removed *ERLIN2*, *DIRAS3*-Ex2 and *IGF1R* from the list of the gDMRs, because these regions showed highly unstable methylation levels among our controls and in an independent cohort of 2664 individuals (Table S3; ref. [29]). Concerning the GNAS locus, all its iDMRs were considered, because although they are contiguous, their methylation level was not necessarily homogeneous among the patients. A similar criterion was applied to include both gDMRs of the ZNF331 locus. Also, we did not remove the GNAS-NESP:TSS sDMR, because it is clinically associated [4]. By analysing the mean methylation level of the remaining 25 iDMRs by hierarchical clustering and exploring the variability of the dataset by PCA analysis, we found that the controls were well separated from the patients, and these were distinguished into two subgroups (Fig. 1a, b). We identified 35 patients belonging to cluster 2 (C2) and 29 patients belonging to cluster 3 (C3) (Fig. 1c). We then applied the definitions of MLID proposed by Ochoa and collaborators (one clinically associated DMR affected or two non-clinical DMRs affected in addition to the primary epimutation; ref. 10) and those proposed by Mackay and collaborators (two non-contiguous clinically associated DMRs affected, ref. 4) to classify our patients. As proposed, the affected contiguous iDMRs were counted as one for MLID definition [4]. For this purpose, we defined an iDMR as affected if its methylation level deviated from the mean methylation level of the controls by 10% and 3 standard deviations or more. By applying this parameter, we found that all the cases meeting the Ochoa criteria and all the cases meeting the Mackay criteria but three were in the C3 group, while all the non-MLID cases were in C2 (Fig. 1a, Table S4). Significantly, the 13 cases in which a maternal SCMC gene variant was identified were in C3 (Fig. 1a, see also below).

Because the chromosome 14q32 *MEG3/DLK1*:IG gDMR was not covered by our assay, we looked at the methylation level of its secondary *MEG3*:TSS-DMR, whose LoM may be found in MLID patients [4]. Significantly, we found that the three patients with *MEG3*:TSS-DMR LoM as determined by MS-MLPA and Epic array were in the C3 group (Table S4). Similarly, we looked at the methylation of the *ZDBF2/GPR1*:IG and ZNF597:TSS sDMRs and found that 6/7 patients with *ZDBF2/GPR1*:IG hypermethylation and 4/4 patients with ZNF597:TSS hypermethylation were in C3.

We then compared the effectiveness of the methylation array and MS-MLPA to identify the MLID cases. Although these two assays similarly classified

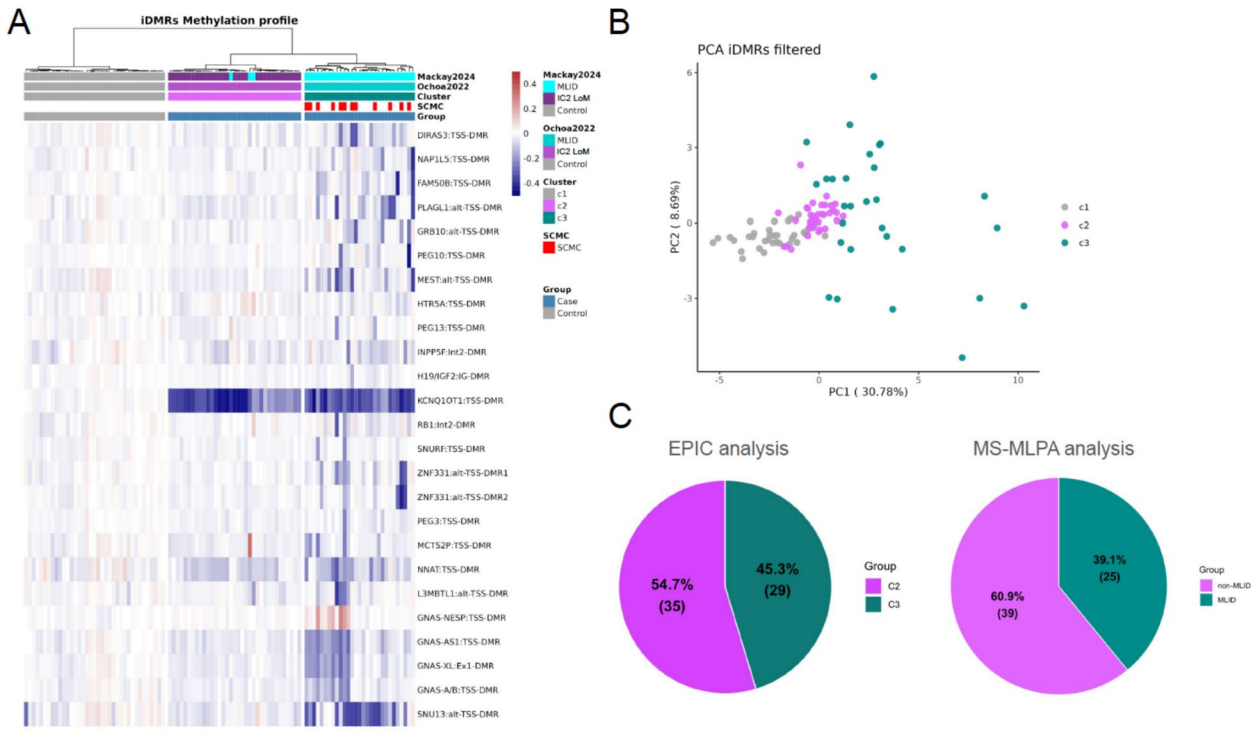

Fig. 1 Methylation profile of iDMRs in BWS patients and control individuals. **A** Heatmap of normalized iDMR methylation beta-values, representing the delta beta values (Δbeta) calculated by subtracting the mean of the controls from each sample. Rows represent individual iDMRs, and columns represent different case samples. Annotations at the top of the heatmap indicate classification of cases according to MLID diagnosis following the criteria of Mackay et al. (2024) and Ochoa et al. (2022), separation in hierarchical clusters (C1, C2, C3), presence of damaging maternal variants in SCMC genes and diagnostic group (Case and Control). In the range colour, blue indicates hypomethylation and red indicates hypermethylation with respect to the mean of the controls. **B** Principal component analysis (PCA) of the samples shown in A based on iDMRs methylation. The PCA plot in the PC1–PC2 space explains 30.78% and 8.95% of the variance, respectively. The dot colour indicates the cluster identity (C1: grey, C2: magenta, C3: teal). **C** Proportional distribution of cases as diagnosed by EPIC methylation array and MS-MLPA. Pie charts representing the proportions of cases separated in cluster C2 and C3 after EPIC array analysis (left) and the cases with MLID or single-locus IC2 LoM based on MS-MLPA methylation analysis (right)

the majority of them, the methylation array allowed us to identify four further cases, which all clustered in C3 (Fig. 1c and Table S5). The iDMRs affected in these 4 cases included *SNU13*:alt-TSS, *FAM50B*:TSS, *MCTS2P*:TSS, *PEG10*:TSS, *DIRAS3*:TSS, *NNAT*:TSS and *ZNF331*:alt-TSS, which are loci not covered by the MS-MLPA assay. To rule out the possibility that the iDMR methylation defects in these four cases were caused by CNVs, we determined the copy number of the imprinted loci by using the methylation array data. Specifically, we employed the conumee 2.0 tool, which detects CNVs with a minimum size of 50 kb and at least 15 probes covered. This analysis revealed no CNV at any tested iDMRs (Fig. S2). In summary, the EPIC methylation array analysis allowed to distinguish two subgroups of BWS patients with different iDMR methylation patterns, one of which was highly enriched in cases with MLID and maternal SCMC gene variants.

Distribution of iDMR methylation in the patient cohorts

Comparison of individual patients with the mean of the controls showed many more differentially methylated iDMRs in the C3 than in the C2 group (Table S4). All iDMRs were affected, but their methylation patterns were not consistent since they were often differentially methylated in different patients. The mean of affected iDMRs was six, ranging from two to fourteen per patient. In addition to *KCNQ1OT1*:TSS, the most frequently affected loci were *GNAS-AS1*:TSS (48%), *GNAS-XL*:Ex1 (41%) and *PLAGL1*:alt-TSS (35%) among the clinical iDMRs (Fig. 2a), while *SNU13:alt-TSS* was the most frequently altered locus (55%) among the non-clinical iDMRs (Fig. 2a; Table S4). In general, LoM was much more frequent than GoM, as LoM was detected at 24 iDMRs, while GoM only at *GNAS-NESP*:TSS sDMR (Fig. 2a; Table S4) likely as consequence of hypomethylation of the cognate gDMR. In the C2 group, 32 out of 35 cases showed

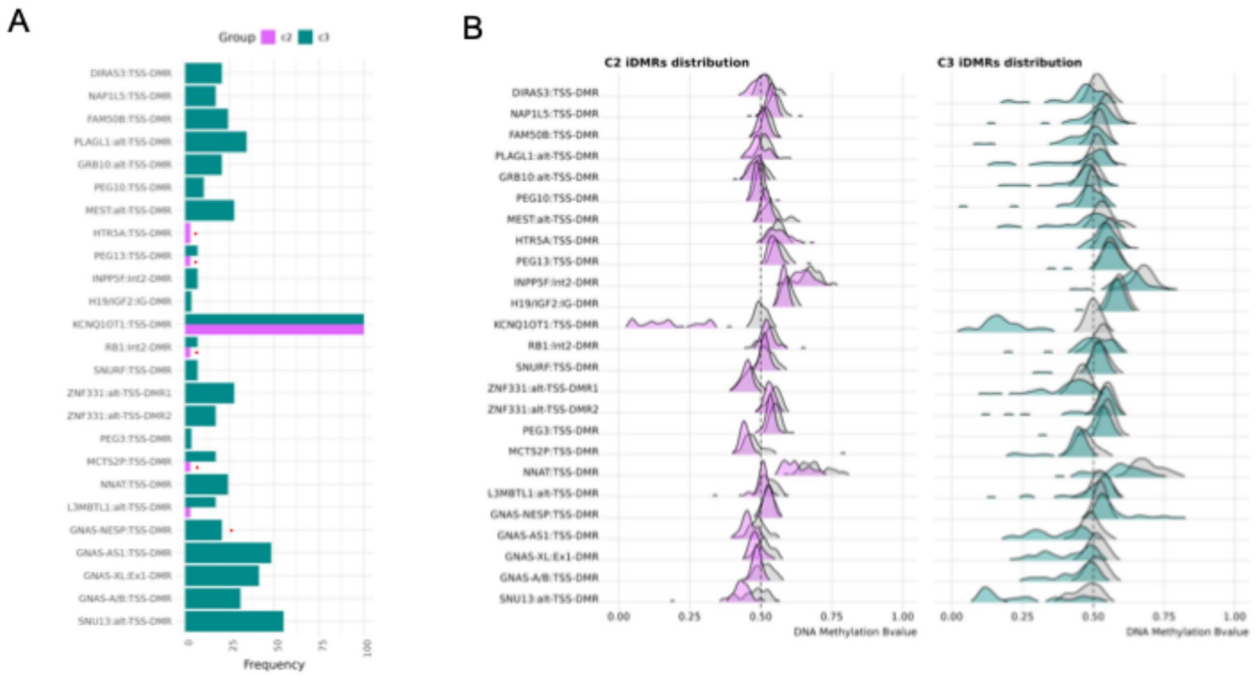

Fig. 2 Frequency of iDMR methylation changes and distribution of iDMR methylation levels in C2 and C3 patient groups and controls. **A** Barplot showing the frequency of methylation changes (difference of at least 10% from the mean of the controls and ± 3 standard deviations) for each iDMR in the C2 (magenta) and C3 (teal) patient groups. The red dots indicate that the iDMR is hypermethylated, while their absence indicates hypomethylation. **B** Density plots displaying the methylation beta-values for each iDMR in the C2 (magenta) and C3 (teal) patient groups and in the controls (grey)

no affected iDMR in addition to *KCNQ1OT1*:TSS. Only 3 cases behaved differently, showing heterogenous hypermethylation of *PEG13*:TSS, *MCTS2P*:TSS, *HTR5A*:TSS and *RB1*-Int2 and hypomethylation of *L3BTL1*:alt-TSS in one case (Table S4). According to the criteria proposed by Mackay and collaborators [4], these 3 cases should be classified as MLID (Fig. 1a).

To better compare the iDMR methylation changes in the C2 and C3 cases, we plotted the distributions of the mean methylation values detected in these two patient groups and in the controls (Fig. 2b). Consistent with their allele-specific methylation, most iDMRs showed a peak of methylation values close to 50% in the controls. However, these distributions were generally shifted or more elongated towards lower methylation values in the C3 than in the C2 cases. The only exceptions were represented by the *GNAS-NESP*:TSS sDMR that gained methylation in several C3 patients and *KCNQ1OT1*:TSS that was hypomethylated in both C2 and C3 cases. The methylation level of all the C3-specific hypomethylated iDMRs was intermediate between 0 and 0.5, suggesting the presence of mosaic methylation disturbances (Table S4). Consistent with these observations, we observed that almost all iDMRs showed higher variability of their methylation values in the C3 group compared to the controls and C2 patients, but only *KCNQ1OT1*:TSS displayed higher methylation variability in the C2 patients compared to the controls (Fig. S3). Thus, all tested gDMRs were variably affected by mosaic hypomethylation in the C3 patient group, while only *KCNQ1OT1*:TSS was consistently affected in C2.

Analysis of DNA methylation variability in the whole genome

Once established that the methylation changes involved all the imprinted loci in the C3 group, we asked if any methylation disturbance affected the rest of the genome in these patients. For this purpose, the methylation analysis was extended to all the CpGs of the genome covered by our assay. We first looked at the differentially methylated positions (DMPs) in C2 and C3 cases compared to controls, by using very stringent criteria (at least 10% mean methylation difference and adjusted p value < 0.001). We found 11,993 DMPs, of which 25 located in imprinted loci, in C2, but only 751 DMPs, of which 137 imprinted, in C3 (Fig. 3a, Table S6). 498 DMPs were in common and showed the same methylation pattern in C2 and C3 (Fig. 3b). Also, in both C2 and C3 the DMPs were distributed across all chromosomes and genomic elements (Fig. 3c). Chromosomes 11 and

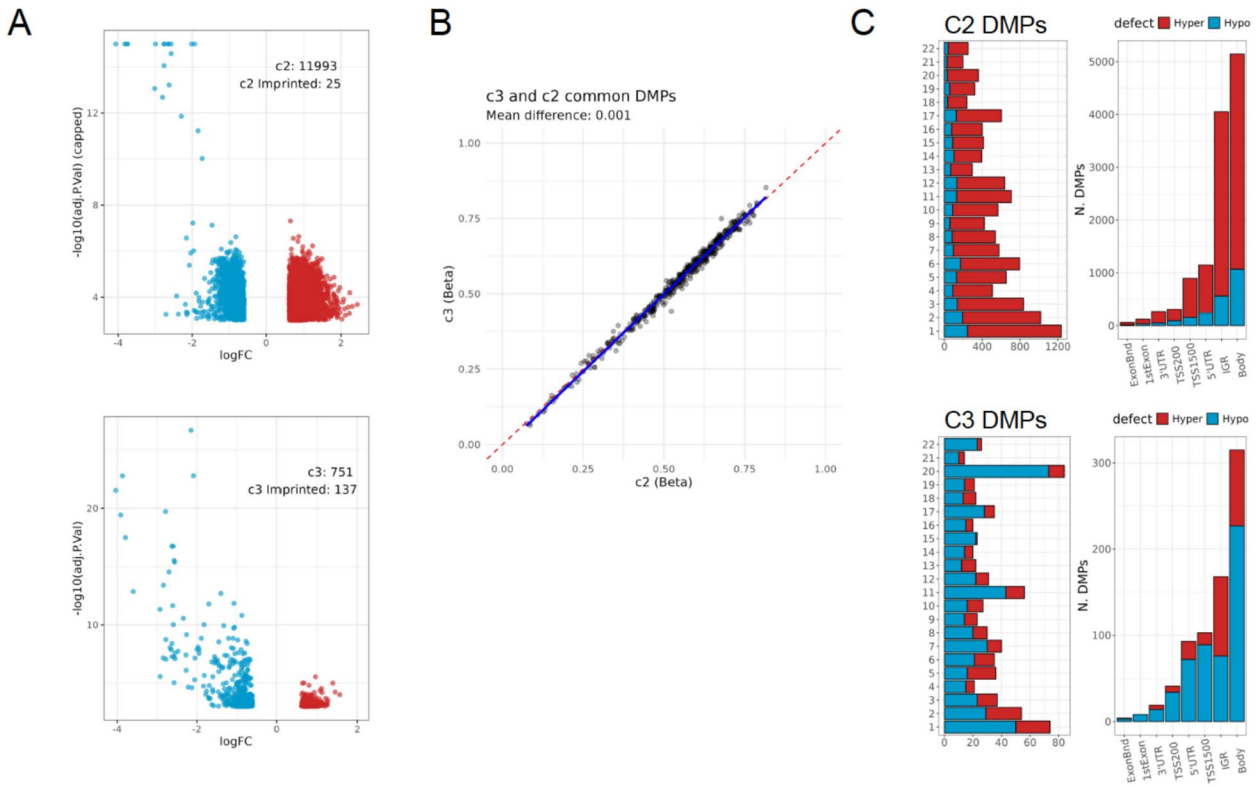

Fig. 3 Analysis of Differentially Methylated Positions (DMPs) between C2 and C3 patients groups and controls. **A** Volcano plots of DMPs in the C2 (upper panel) and C3 (lower panel) patient groups. Each point represents a CpG position, with the *x*-axis showing the log fold change (logFC) in methylation and the *y*-axis representing statistical significance (– log10 adjusted *p* value). The total number of DMPs identified and the DMPs mapping to imprinted loci are indicated for each patient groups. Hypermethylated DMPs are in red and hypomethylated DMPs in blue. **B** Methylation level of the DMPs shared between the C2 and C3 patient groups. Scatterplot displaying the mean methylation profile of each common DMPs in C3 (*Y*-axis) and C2 (*X*-axis). **C** Genomic distribution of the DMPs in C2 (upper panel) and C3 (lower panel) patient groups. Bar plots showing counts of hyper-(red) and hypo- (blue) methylated CpGs mapping to different chromosomes (1–22, *Y*-axis) and genomic elements: exon boundaries (ExonBnd), at the 1stExon, 3'UTR, 5'UTR or Body, 200 bp or 1500 bp from the TSS (TSS200, TSS1500) and intergenic regions, (IGR) on *X*-axis

20 were relatively more represented in C3, because of the presence of more probes in the imprinted domains located in these chromosomes. The majority of the DMPs were hypermethylated in C2 but hypomethylated in C3 (Fig. 3c). Among the gene categories affected by these methylation changes, only imprinted genes were significantly represented in C3, while genes involved in several metabolic and signalling pathways were affected in C2 (Fig. S4a). A few genes, such as the hypomethylated *FOXP1* and *BCL11B,* both associated with intellectual disability, were affected in both C2 and C3 patients (Fig. S4b).

Finding more DMPs in the C2 than C3 patients was unexpected. Because iDMR methylation changes were highly variable among the C3 patients, we reasoned that consistent methylation changes could also be masked by higher variability in the rest of the genome in this patient group. To test this hypothesis, we looked for the CpGs whose methylation level was more variable (p value < 0.05) in the patients than in the controls, and we defined them as differentially variable positions (DVPs). In this case, we found 10,545 DVPs in C3 but only 651 DVPs in C2, of which 431 were common to both patient groups (Fig. 4a, Table S7). 346 and 22 DVPs were mapped to the iDMRs in the C3 and C2 groups, respectively (Fig. 4a). The most variable DVPs were in imprinted loci but located only in the *KCNQ1OT1*:TSS-DMR in C2 patients and spread across several chromosomes in C3 patients (Fig. 4a, b). The majority of the other DVPs were distributed on all chromosomes and located primarily in gene bodies and intergenic regions, as well as more than 2500 promoters in C3 patients (Fig. 4b). The KEGG analysis of the loci where the DVPs were mapped indicated that two cancer-related pathways, the focal adhesion and other signalling pathways were specifically affected in C3 patients. Instead, the calcium signalling, cholinergic synapse and thyroid hormone signalling pathways instead were affected in both C2 and C3 patient groups (Fig. 4c, d).

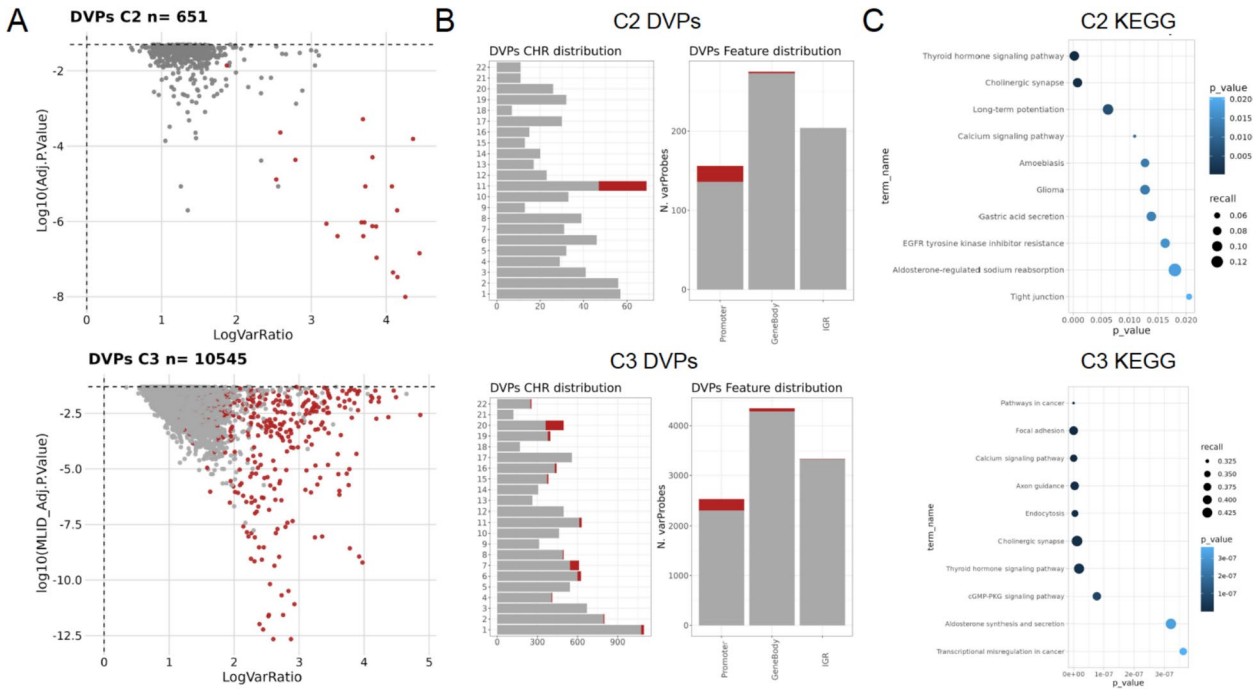

Fig. 4 Analysis of Differentially Variable Positions (DVPs) between C2 and C3 patients groups and controls. **A** Volcano plots of DVPs in the C2 (upper panel) and C3 (lower panel) patient groups. Each point represents a CpG position, with the *x*-axis showing the log variance ratio (logVarRatio) and the *y*-axis showing – log10 adjusted *p* value. The DVPs overlapping an iDMR are indicated by red dots. **B** Genomic distribution of DVPs in C2 (upper panel) and C3 (lower panel) patient groups. Bar plots showing counts of DVPs mapping to different chromosomes and genomic elements. The DVPs mapping to the iDMRs are indicated in red. **C** KEGG pathway enrichment analysis of genes associated with DVPs in C2 (upper panel) and C3 (lower panel) patient groups. Dot size reflects recall (gene ratio), and colour indicates – log10 (*p* value)

We sought to analyse the C3-specific DVPs in more details. First, we classified the affected CpGs according to their mean methylation level in the controls and found that the DVPs were significantly more represented among the CpGs with intermediate methylation level ($40\% < x < 60\%$ and $60\% < x < 80\%$) than among the CpGs with lower (< 40%) and higher (> 80%) methylation levels in the controls (Fig. 5a). To look for the genomic loci more affected by methylation variability, we annotated the C3-specific DVPs to chromosomal bands and found a statistically significant enrichment at chr5q31.3, where a cluster of protocadherins (PCDHs) is located (Fig. 5b and Fig. S5). In addition, we found significantly higher methylation variability in the gene body and promoter region of several genes associated with cancer (e.g. *B4GALT5*, *SLC12A9* and *SNED1*) (Fig. 5d, Table S8). In summary, many CpGs located throughout the genome and preferentially bearing intermediate methylation levels in the controls displayed variable methylation changes in the C3 patients.

Genetic analysis

Gene variants were investigated by WES in 35 mothers of BWS patients, including 14 mothers of the C2 group and 21 mothers of the C3 group. All variants predicted to be damaging by either PolyPhen or SIFT exhibited comparable distribution and mean in the C2 and C3 mother groups (Fig. 6A, B). Conversely, variants in the SCMC genes were identified 11 C3 patient mothers, but none of the C2 patient mothers (Table S10). The variants affected the *PADI6* (5 cases), *NLRP5* (3 cases), *NLRP2* (2 cases), *KHDC3L* and *NLRP4* (1 case each) genes and were present in homozygosity or compound heterozygosity in 7 cases and in heterozygosity in the remaining ones. Ten of these variants were previously described [25–27]. The novel *PADI6* Val562Met variant was identified in the present study. Moreover, we found several potentially damaging variants of further MEGs [30, 31] (Table S11) in the mothers of our patients (Table S12). The affected genes were associated with female fertility and embryo development, and their encoded proteins were not demonstrated to be part of the SCMC so far. Similar to the SCMC variants, all the MEG variants were more enriched in the mothers of the C3 patient group than in the C2-mothers, and the majority of affected genes were C3-specific (Fig. 6B and Table S12). Most of the non-SCMC gene variants were present in heterozygosity, but two genes (*UBA7* and *TP73*) were affected in more than one patient

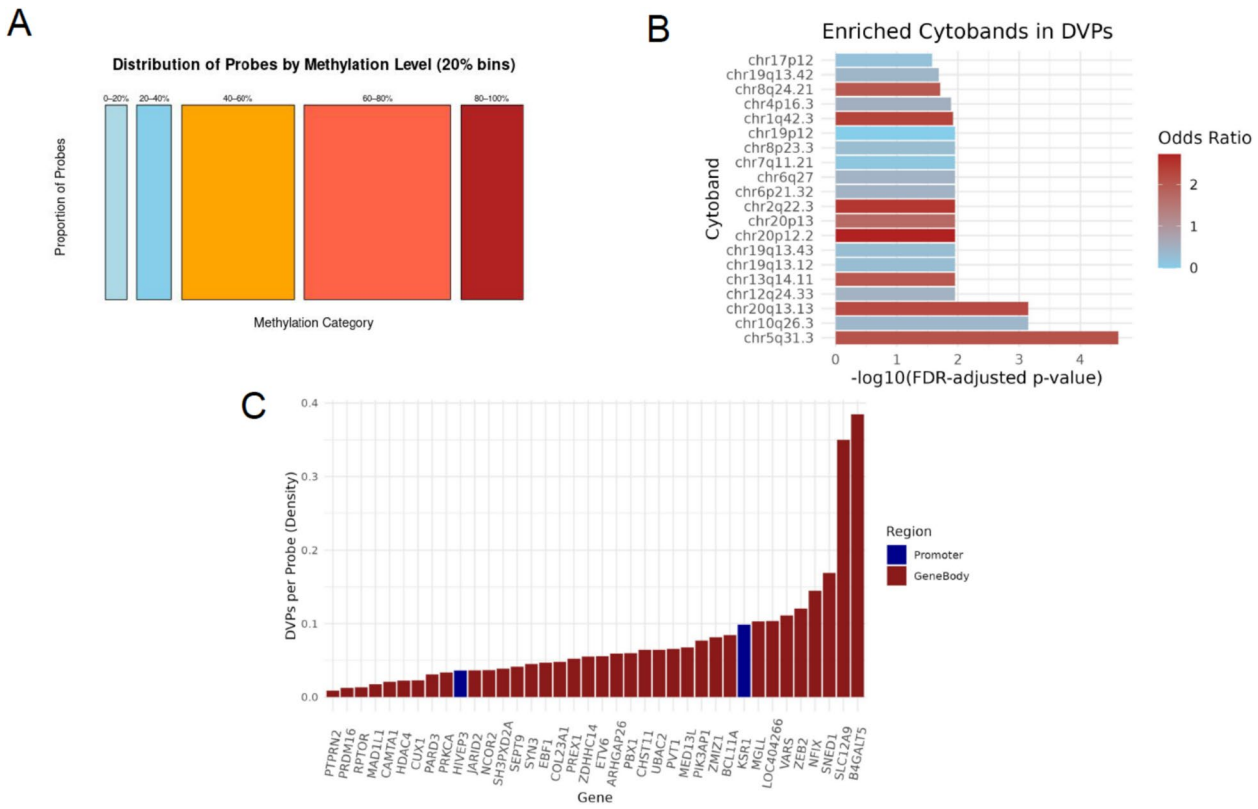

Fig. 5 Genomic characterization of the DVPs of the C3 patient group. **A** Mosaic plot showing the enrichment of the C3-DVPs in bins characterized by different CpG methylation in the control individuals. **B** Bar plot showing significantly enriched cytobands containing the C3-DVPs. The x-axis indicates −log10(FDR-adjusted *p*-values) and colour reflects the odds ratio, highlighting enrichment strength. Redder bars represent stronger enrichment (odds ratio > 2). **C** Bar plot showing the density of DVPs in the promoter and body of genes covered by the array. The density is calculated as the ratio between the number of DVPs and the total number of covered CpGs for that gene. To look for further DVPs that were distributed as contiguous blocks or groups of CpGs, we identified 2 kb windows with > 3 DVPs. Besides several imprinted regions, we found DVP clusters within the gene body of *HOXB6*, the promoter region of *LHX8*, the gene body of *VARS1*, and both the gene body and promoter of *FOXI2* (Table S9)

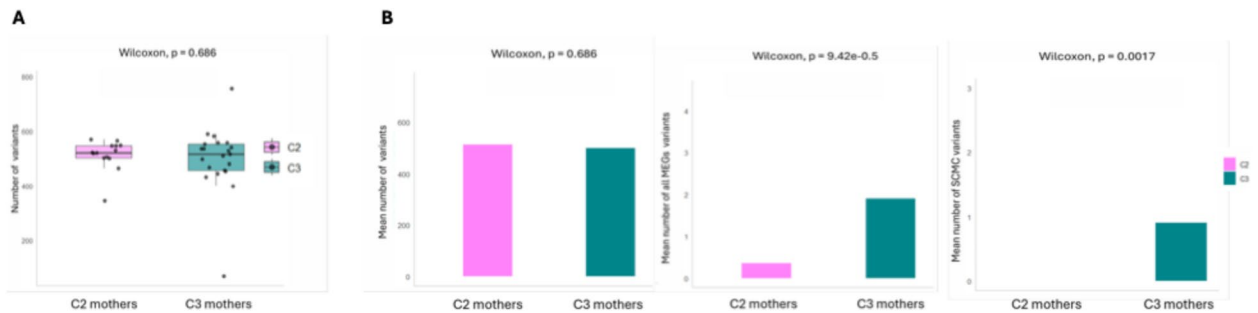

Fig. 6 Occurrence of potentially damaging variants identified by WES and in silico prediction in the maternal genomes of C2 (teal) and C3 (magenta) cases. **A** Boxplots showing the distribution of the number of all variants in C2 and C3 mother groups. **B** Barplots displaying the mean number of all variants (left), maternal-effect gene variants (MEGs; middle), and SCMC variants (right) in the C2 and C3 mother groups

and *UBA7* variants were present in compound heterozygosity in one case. Thus, the mothers of the C3 patients carried more potentially damaging variants in the SCMC and other MEGs than the mothers of the C2 patients.

Clinical findings

The clinical scoring system proposed by Brioude et al. [6] was applied to our patient cohort (Table S1). Overall, the patients displayed a score ranging between 3 and 11, with

no significant difference between the C2 and C3 groups (median 6 and 7 in C2 and C3, respectively). Also, the frequency of typical BWS features or other less common clinical features did not differ significantly between the two patient groups (Fig. S6 and Table S1). Furthermore, no significant difference in the prevalence of twinship nor gender discrepancy in C2 and C3 was observed.

Maternal reproductive history was documented for 60 out of 61 patient families (Table S1). In 4/27 families of the C3 group but none of the C2 group, the mother experienced multiple (>=3) miscarriages and/or had more than one affected child (Fig. 7a). All these women carried MEG variants, and specifically biallelic pathogenic variants in the SCMC genes. Overall, eight patients were conceived with the help of assisted reproductive technology (ART), and two of them carried maternal SCMC variants. However, ART prevalence did not differ significantly between the C2 and C3 groups (Fig. 7b). Data

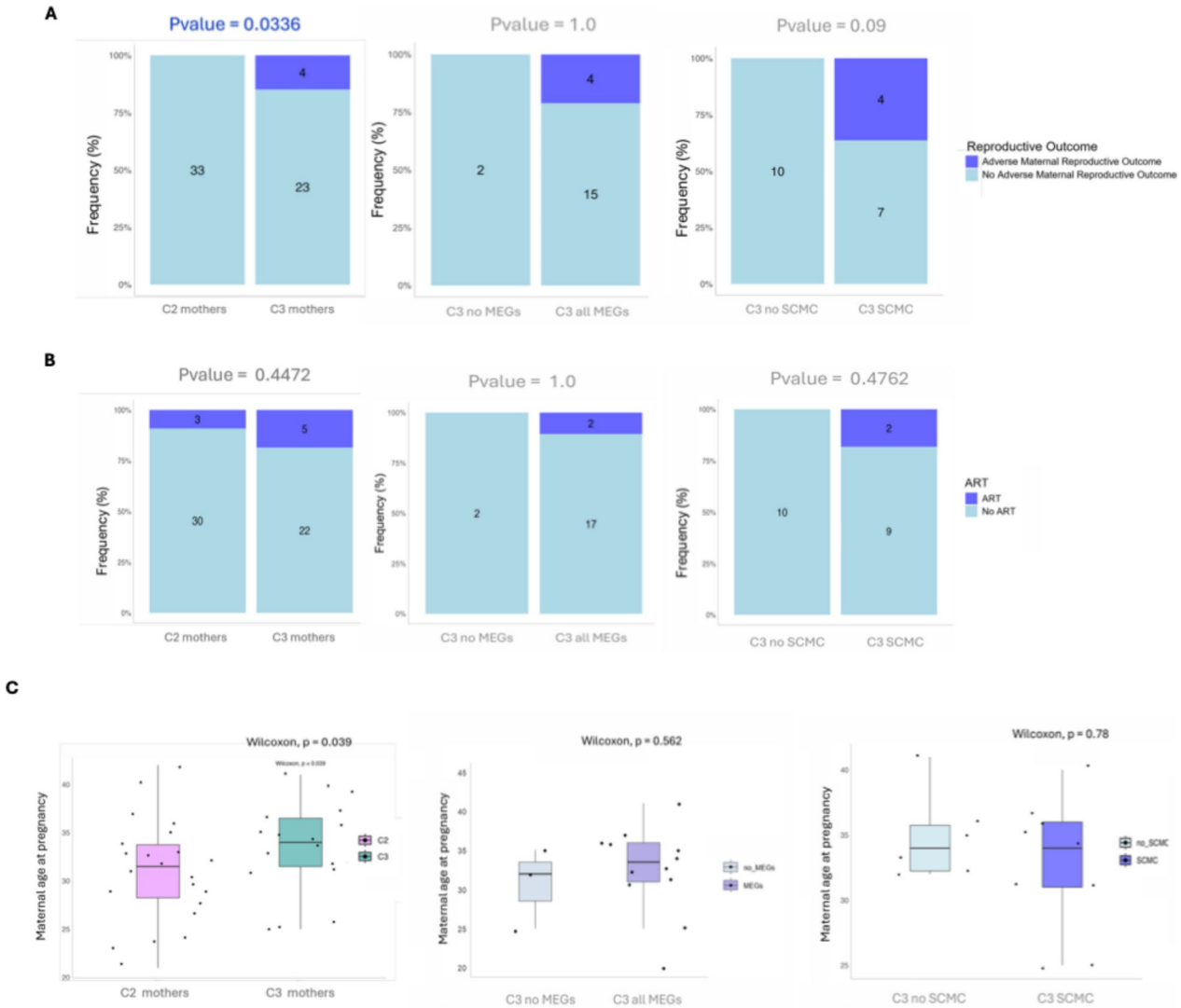

Fig. 7 Familial recurrence and maternal reproductive history in the C3 and C2 groups. **A** Percentage of families with (dark blue) or without (light blue) adverse reproductive outcomes in the C2 and C3 mother groups (left panel), in the C3 mother subgroups with or without MEG variants (middle panel), and with or without identified SCMC gene variants (right panel). **B** Percentage of families in which the proband was conceived with the help of ART (dark blue) or was naturally conceived (light blue) in the C2 and C3 mother groups (left panel), in the C3 mother subgroups with or without MEG variants (middle panel), and with or without identified SCMC gene variants (right panel). **C** Box plot displaying the maternal age at pregnancy in the C2 (pink) and C3 (green) mother groups (left panel), in the C3 mother subgroups with (dark violet) or without (light violet) MEG variants (middle panel), and with (dark blue) or without (light blue) identified SCMC gene variants (right panel). The median age is indicated by a dashed line. Adverse reproductive outcomes: multiple (> 1) affected children or recurrent (> = 3) pregnancy loss; No adverse reproductive outcomes: 1 affected child or pregnancy loss < = 2

concerning maternal age at pregnancy were available for 41 conceptions, including 22 mothers of the C2 group and 19 mothers of the C3 group (Table S1). We observed that maternal age was significantly higher (median: 34 vs 31) in the mothers of the C3 group than those of the C2 group (Fig. 7c). However, no correlation was observed between age at pregnancy and the presence of MEG variants or SCMC gene variants within the C3 group (Fig. 7c). In summary, no difference in clinical features was evident between the C2 and C3 cases. However, the mothers of the C3 patients experienced more miscarriages, had higher recurrence risk in their progeny and were older than the mothers of the C2 patients.

Discussion

MLID is an interesting but so far poorly understood phenomenon occurring in a subset of BWS patients. Indeed, the paucity of cases reported and the limitations of the diagnostic methods have hampered the definition of the underlying molecular mechanisms so far. In this study, we used whole-genome methylation data to distinguish the cases with MLID (cluster 3) from those with single-locus IC2 LoM (cluster 2), and found that all covered gDMRs and an elevated number of non-imprinted loci were subjected to variable methylation changes in the former group of patients. Moreover, we demonstrated that the mothers of the patients with MLID carried more variants in maternal-effect genes and had a higher age at pregnancy than those of the single-locus IC2 LoM cases. These results suggest that MLID arises as a consequence of defective maintenance of whole-genome methylation that, in turn, is caused by genetic and non-genetic alterations of maternal factors.

Most of the studies on MLID performed so far were restricted in the number of loci analysed, and it is not clear if all imprinted genes are affected in these cases [4]. The whole-genome methylation analysis performed in this study demonstrated that most, if not all, of the gDMRs may be altered in a subset of BWS patients, and the methylation levels of all these loci are useful to distinguish the cases with MLID from those with single-locus methylation changes. Only a few imprinted loci should be excluded from this analysis because they display too high variability in the control individuals. Thus, a diagnostic assay investigating more loci than the ones analysed by the currently used MS-MLPA kit is advisable. Also, our study suggests limiting the diagnostic test to gDMR LoM, because these methylation changes appear more specific than gDMR GoM to MLID cases. However, hypermethylation of the paternally methylated and secondary *ZDBF2/GPR1*:IG and *ZNF597*:TSS DMRs and LoM of *MEG3*:TSS are frequent in MLID cases and may be used as proxy of LoM of the cognate gDMRs in case these are not well covered by diagnostic assays.

When the whole epigenome was analysed, we found > 15-fold more differentially methylated CpGs in the cases with single-locus IC2 LoM than in the cases with MLID. Only a few hundred differentially methylated CpGs were in common between the two cohorts and mapped in two genes associated with intellectual disability. However, when we looked for CpGs with more variable methylation levels, we found the opposite scenario, a 16-fold difference between the MLID and the single-locus IC2 LoM cases. We interpreted this finding as if higher variability in CpG methylation was masking the differences in mean methylation level between the MLID patients and the controls. Interestingly, we found that one of the loci more significantly affected by methylation variability was a cluster of protocadherin genes on chromosome 5q31.3 that is controlled by DNMT3b-dependent de novo CpG methylation during development [32, 33]. Further loci affected by high methylation variability included several cancer-related genes, such as *SNED1* [34] that is associated with breast cancer, *SLC12A9* associated with colorectal cancer and glioma [35, 36] and *B4GALT5* [37] associated with ovarian cancer. In addition, the methylation of the promoter of *KSR1,* which is involved in the RAS-RAF-MEK-ERK cascade and TP53 control [38], is frequently altered in patients with MLID. Also, *LHX8* and *VARS1*, which are associated with brain development [39, 40] and *FOXI2* and *HOXB6*, which play crucial roles in embryogenesis [41, 42], were characterized by high methylation variability in the MLID patients. In the mouse, inactivation of maternal SCMC genes leads to a reduction of UHRF1, which is a key molecule for DNA methylation maintenance in early embryogenesis [7, 8]. In humans, a similar UHRF1 reduction may lead to inefficient or disordered re-methylation of many genomic CpGs including those of the gDMRs during development. Intriguingly, the CpGs that are more affected by methylation variability display intermediate methylation level and their methylation maintenance is likely more vulnerable.

Complete inactivation of the maternal SCMC genes leads to early development arrest in both human and mouse [8]. However, maternal hypomorphic variants of these genes also segregate in women with progeny affected by imprinting disorders with MLID, suggesting that residual SCMC activity is sufficient to complete embryo development but interferes with correct imprinting methylation maintenance [4]. Consistent with these studies, we demonstrated that maternal-effect variants affecting the SCMC genes were highly enriched in the BWS cases with MLID when compared with the BWS cases with single-locus IC2 LoM. Although the cases

were not sufficient to reach statistical significance, our results also suggest that the women carrying the SCMC gene variants have higher recurrence risk (progeny with > 1 affected child or 1 affected child + ≥ 3 pregnancy losses) than the C3-mothers without these variants. We found potentially damaging variants in further maternal-effect genes that may be characterized by lower penetrance and act synergistically in the latter group of patients. Among the non-SCMC genes, it is worth mentioning *UBA7* and *TP73* that are affected in multiple C3 cases. *UBA7* (also known as *UBE2*) encodes an ubiquitin-activating enzyme, and the rare damaging Pro712Ser variant is present in the mother of three C3 patients. A second possibly damaging *UBA7* variant (Val509Met) is present in one of these women, in compound heterozygosity. Protein ubiquitination is required for maternal to zygotic transition and early development of embryos [43]. Finally, *TP73* is a maternally expressed imprinted gene encoding a member of the p53 transcription factor family involved in the maintenance of germ-line genomic integrity and whose expression is lowered in aged human oocytes [44]. These data suggest a possible role of these genes in the epigenetic reprogramming of early embryos, although their possible involvement in the aetiology of MLID requires investigation of larger cohorts of patients. Our findings underscore the importance of screening for MEG variants in MLID families for more accurate counselling on recurrence risks and reproductive challenges.

Differently from previous reports suggesting that methylation defects in addition to those of chromosome 11p15.5 may modify the typical clinical picture of BWS [25, 26, 45], no significant difference in clinical signs between C2 and C3 patient groups were identified (Fig. S6). Indeed, atypical features, including neurodevelopmental delay and feeding difficulties, were found in both C2 and C3 patients, with similar frequencies. Also, locus- specific epigenotype-phenotype correlations appear complex in our cohort. For example, PLAGL1 and MEST hypomethylation were associated with variable growth outcomes, and GNAS hypomethylation was not strictly associated with hypocalcemia**.** Concerning H19/IGF2:IG-DMR LoM, we only have one case in our cohort who did not show growth defects at birth, potentially suggesting compensatory mechanisms with concurrent IC2 LoM [25]. However, the other cases without macrosomia at birth did not display any methylation abnormality at this locus. These findings indicate that the relationship between specific iDMR methylation patterns and clinical phenotypes in MLID is more complex than simply additive effects, potentially involving tissue-specific mechanisms.

Both clinical and non-clinical iDMRs showed altered methylation in the patients from families harbouring MEG variants and/or with history of adverse reproductive outcomes (Table S4, Table S10, Table S12). Although our findings indicate no apparent correlation between the presence of MLID and clinical features, given the limited sample size of our study, we cannot definitively conclude that including loci beyond the traditionally recognized clinically associated loci in molecular diagnostics is unwarranted. Analysis of additional cohorts for longer time periods is necessary in the future to increase our understanding and improve diagnostics and management of MLID.

The association between ART and MLID is controversial [4, 45]. We did not find any difference in the frequency of individuals conceived with the help of ART between our patient cohort with MLID and the cohort with single-locus IC2 LoM, suggesting that larger cohorts of patients should be studied to reach conclusive results on these issues.

An interesting finding of our study is the association of maternal age and risk of MLID. Expression of SCMC genes and encoded proteins is affected in aged oocytes of humans and animal models [44]. Because these proteins are specifically synthesized in oocytes, they may be stored for several decades until they are transferred to the human embryo after fertilization. This long-term storage can easily expose them to oxidative damage or insult by environmental agents. Although maternal age differed between the C2 and C3 groups, we did not find significant differences between the C3 cases with MEG/SCMC variants and the C3 cases in which such variants have not been identified. This suggests that oocyte ageing interacts with and increases the penetrance of maternal gene variants in the former group of patients.

A limitation of this study is the insufficient coverage of some of the iDMRs by our assay. This may have hidden some epigenotype-phenotype correlations in our cohort. Future studies employing methods with higher coverage of imprinted loci should solve this problem.

Conclusions

Our study supports a model in which environmental factors and trans-acting genetic variants interact in aged oocytes to cause MLID in the progeny. In our model, MLID originates as a defect of oocyte proteins leading to disordered DNA methylation maintenance in the pre-implantation embryo. We predict that methylation changes occur with high variability in the whole genome and affect both imprinted and non-imprinted loci in an apparently stochastic manner. Although we did not find any correlation between MLID and clinical phenotype in our patients, it is important to remember that we studied only the BWS cases during childhood. Because several loci with relevant biological functions are affected in

the cases with MLID, it will be important in the future to understand if and how these epimutations impact the health of these individuals during adulthood.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13148-025-01971-4.

Additional file1

Additional file2

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Author contributions
Fr.C., L.P., Fl.C. and A.R. designed experiments and interpreted results. Fr.C., L.P., E.D'A., C.G., Ab.S. and An.S. performed experiments. B.H.M. and C.A supervised bioinformatics analyses. A.M., G.B.F., G.S., G.G., E.DiM., C.R., L.T., C.P., I.S., J.A.T. and P.L. provided clinical cases. Fr.C., L.P., F.C. and A.R. wrote the manuscript with input from all authors. All authors reviewed the manuscript.

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Availability of data and materials
The raw and processed files of the methylome array are deposited in GEO under the accession number GSE 297935.

Declarations

Ethics approval and consent to participate
The study was approved by the ethical committee of the University of Campania Luigi Vanvitelli.

Consent for publication
The family agreed for publication by signing an informed consent template.

Competing interests
The authors declare no competing interests.

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