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Investigation of Multilocus Imprinting Disturbance (MLID) in 101 Beckwith-Wiedemann Spectrum patients

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ABSTRACT

Beckwith-Wiedemann spectrum (BWSp) is an overgrowth disorder caused by both genetic and epigenetic defects within the 11p15.5 chromosomal region. The most common cause of BWSp is DNA methylation anomalies in two imprinting control regions (ICR1, the telomeric centre that includes *H19*/*IGF2*:IG-DMR and ICR2, the centromeric centre that includes *KCNQ1OT1*:TSS-DMR) located within the 11p15.5 locus. Previous studies demonstrated that a subset of BWSp patients had methylation defects extending beyond 11p15.5 to other chromosomal loci, an entity known as multilocus imprinting disturbances (MLID).

In this study, the multilocus methylation status of 101 BWSp patients was analysed by both various methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA-E034) and methylation microarrays. MS-MLPA-ME034 detected MLID in 16 (15.8 %) of the patients, which increased to 59 (58.4 %) using methylation microarrays. ICR2 hypomethylation was observed in all MLID cases, and 28 imprinted differentially methylated regions (DMRs) were additionally detected. Recurrent loci associated with the genes such as *GNAS*, *MEST*, and *DIRAS3*, previously reported in MLID patients, were also observed as hypomethylated in our cohort. Seven out of the 48 (14.6%) MLID-BWSp patients with complete data on type of conception were born following assisted reproductive technologies (ART), indicating an appreciable proportion of MLID among ART-conceived pregnancies. This study underscores the value of genome-wide methylation analyses in revealing molecular complexity, improving diagnostic accuracy, and informing prenatal care in BWSp with MLID. Future research should further explore the long-term clinical implications of MLID and the underlying molecular mechanisms.

KEY WORDS: Beckwith-Wiedemann spectrum, BWSp, methylation, Multilocus Imprinting Disturbances, MLID, microarray, MS-MLPA.

INTRODUCTION

Beckwith-Wiedemann spectrum (BWSp, MIM #130650) is an overgrowth disorder characterized by a highly variable spectrum of clinical features, including macroglossia, macrosomia, abdominal wall defects (such as omphalocele, diastasis recti or umbilical hernia), neonatal hypoglycaemia and predisposition to tumour development, among many others (1). The estimated prevalence of BWSp is approximately 1 in 12,000 live births, although this rate is higher in individuals conceived through assisted reproductive technologies (ART) (2).

BWSp results from both genetic and epigenetic alterations within the 11p15.5 chromosomal region. In 2018, the term Beckwith-Wiedemann spectrum (BWSp) was suggested to cover classical Beckwith-Wiedemann syndrome (BWS) without a molecular diagnosis and BWS-related phenotypes with an 11p15.5 molecular anomaly (3). The 11p15.5 locus encompasses two distinct imprinting control regions (ICRs; ICR1, the telomeric one which includes *H19*/*IGF2*:IG-DMR and ICR2, the centromeric one which includes *KCNQ1OT1*:TSS-DMR), which consist of clusters of genes playing crucial roles in biological processes, especially during embryonic development, such as somatic growth, cell cycle regulation and proliferation (1,2,4). The primary cause of BWSp is DNA methylation defects at these ICRs: over 50% of patients present with loss of methylation (LoM) at the maternal ICR2, 5-10% exhibit gain of methylation (GoM) at the maternal ICR1, and 20-25% shows mosaic paternal uniparental disomy (UPD) within chromosome 11p. Hypomethylation of ICR2 and hypermethylation of ICR1 led to the dysregulation of *CDKN1C* (Cyclin-Dependent Kinase Inhibitor 1C) and *IGF2* (Insulin-Like Growth Factor II), respectively (5,6). Other rarer causes of BWSp include pathogenic variants in *CDKN1C* (5-10%) and large genomic rearrangements involving the 11p15.5 locus (~ 2-3%) (7).

Previous studies demonstrated that some patients with BWSp had methylation abnormalities at other loci additional to the 11p15.5 region (8–10), known as multilocus imprinting disturbance (MLID) (11). The additionally affected differentially methylated regions (DMRs) observed in patients with BWSp are usually associated with the imprinted *PLAGL1*, *GNAS*, *GRB10*, *MEG3*, *PEG3* and *MEST* genes (8,9,12–16). Thus, the term "clinically associated" DMRs (CA-DMRs) refers to those DMR where (epi)genetic changes altering expression are directly associated with a canonical imprinting disorder (11). The underlying molecular mechanism of MLID is not entirely understood; however, monogenic variants in several genes have been identified in mothers of some patients with MLID, including members of the NOD-like receptor protein (NLRP) family (*NLRP2*, *NLRP4*, *NLRP5*, *NLRP7)* and others, *(PADI6* and *KHDC3L*). These genes encode proteins that are part of the subcortical maternal complex (SCMC) and are essential for early embryonic development (11,17). Additionally, other variants have also been identified in children with MLID which can be associated with an increased risk of an imprinting disorder, this includes variants in *ZFP57* and *ZNF445*. Both ZFP57

and ZNF445 are key transcription factors that play complementary roles in the maintenance of genomic imprinting during early embryonic development, and their loss of function has been associated with MLID. ZFP57 is essential for maintaining methylation at many imprinted DMRs after fertilization (18), and biallelic pathogenic variants in *ZFP57* have been directly linked to MLID, especially in patients with transient neonatal diabetes mellitus (19). ZNF445 acts as a primary regulator of imprinting in humans, binding a broad set of imprinted loci and at least one homozygous variant in this gene has been associated with MLID (15,20).

The growing advances in methylation analysis, such as methylation microarrays and methyl-seq, provide a comprehensive approach for examining methylation status across the genome (21–23). These high-throughput technologies offer the potential to uncover novel insights into the complex epigenetic landscape of patients with MLID, allowing the identification of well-known DMRs as well as potentially new regions with aberrant methylation that could influence the phenotype of patients.

In this study, we applied a combination of methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA-ME034; 11 different imprinted loci within seven chromosomal regions) and genome-wide methylation microarray assays (Infinium Methylation EPIC v2.0 BeadChip, Illumina) in 101 BWSp patients, with the aim of identifying the extent of MLID and looking for additional regions with aberrant methylation.

PATIENTS, MATERIALS AND METHODS

Patients

Patients included in this study were selected from the Spanish Overgrowth Syndromes Registry Initiative (SOGRI), (see list in Acknowledgements) which contains more than 2,500 individuals and relatives with overgrowth disorders. All selected patients (N=101) had previously been diagnosed with BWSp based on their clinical findings and subsequent detection of abnormal methylation on the 11p15.5 chromosomal region with the SALSA MS-MLPA probemix ME030 BWS/RSS kit (MRC-Holland, Amsterdam, Netherlands). Eighty-five patients presented with hypomethylation of ICR2 (84.1 %), 13 presented with paternal UPD (12.9 %) and three presented with hypermethylation of ICR1 (3 %) (Tables 1 and 2). This study was approved by the Ethical Committee of the Hospital Universitario La Paz (CEIm PI-446), and informed consent was obtained from all patients and/or their parents/ legal guardians. We reserved the definition "ART" for interventions that involve in-vitro handling of oocytes/embryos such as in vitro fertilization (IVF) treatment and intracytoplasmic sperm injection (ICSI). Intrauterine insemination (IUI) is not formally considered an ART because only the sperm is manipulated (24). We also defined

"classical BWSp" when a BWSp patients had a BWSp score ≥ 4 and "atypical BWSp" when the score was < 4 (3).

MS-MLPA analysis

The SALSA MS-MLPA probemix ME034 Multi-locus Imprinting kit (MRC-Holland, Amsterdam, Netherlands) was applied to identify samples with MLID. It is composed by 47 probes, 5 provide information on the methylation status of the H19/IGF2:IG-DMR, 2 of the IGF2:alt-TSS-DMR, and 4 of the KCNQ1OT1:TSS-DMR. In addition, information on the methylation status of MEST:altTSS-DMR and GRB10:alt-TSS-DMR is provided by 2 probes per DMR, as described in the MS-MLPA specifications. Data analyses were conducted following the manufacturer protocol, defining relative probe signals by dividing each measured peak area by the sum of all peak areas of the control probes for that sample. Each peak's relative probe area ratio was then compared to a DNA control sample (Promega, UK), using Coffalyser.net (MRC-Holland, Amsterdam, Netherlands).

Methylation microarrays

DNA was extracted from whole blood collected from 101 BWSp patients and 41 controls. Control samples were selected based on age and sex matching to the BWSp cohort in order to minimize potential confounding factors. All control subjects were clinically healthy, with no known diseases or molecular alterations, ensuring a reliable baseline for comparative analyses. Bisulfite conversion of the DNA was performed using the EZ DNA Methylation-Lightning™ kit (Illumina, San Diego, CA). Methylation levels were assessed using the Infinium Methylation EPIC v2.0 BeadChip (Illumina, San Diego, CA) microarray, which covers over 930,000 CpG sites. Raw data (*.idat* files) were imported into R (v4.4.1), processed, and normalized using the R package *minfi* (v1.50.0) (25) and subjected to quality control. Probes were filtered out based on the following criteria: detection p-value > 0.01, probes located on the X and Y chromosomes, probes known to contain a single-nucleotide polymorphism (SNP), and probes known to cross-react with other genomic locations. After this filtering step, β-values and M-values, representing the methylation levels at each CpG site, were calculated using *getBeta* and *getM* function from *ENmix* R package (v1.44.3). Sample labelling verification, including sex and age estimation, was conducted using the R packages *wateRmelon* (v2.10.0) (26) and *methylclock* (v1.10.0) (27).

Methylation status of the patients was first evaluated at the CA-DMRs (11) reported by Court et al. (2014) (28). To assess methylation status, the mean β-value and standard deviation (SD) of controls were calculated for each probe. We concluded that patients were classified as having MLID based on their methylation profiles. A standardized score was computed for each probe, and probes were considered significantly hypermethylated if the score was ≥ +3 SD or hypomethylated if the score was ≤ -3 SD relative to the control mean. Patients presenting three or

more probes with scores beyond these thresholds, either hypermethylated or hypomethylated, were designated as MLID. Additionally, we performed a differential methylation analysis to compare methylation profiles between controls and BWSp patients, leading to the identification of differentially methylated CpG sites outside the previously analysed germline DMRs (gDMRs). To visualize these CpGs across the genome, the R package ENmix (v1.40.2) (29) was used. The top differentially methylated CpG sites were used to generate a heatmap with the R library ComplexHeatmap (v2.20.0) (30), allowing the identification of methylation patterns across our cohort beyond the gDMRs.

Statistical analysis of clinical features

Statistical analysis was conducted using SPSS v.25 (IBM Corporation, United States) to assess differences in clinical features. Descriptive analyses included the mean ± SD for continuous variables (age in this cohort) and frequency tables for categorical variables. Categorical variables were expressed as binary values (1 or 0), categorized as "ever" having a given condition versus "never" having the condition. Figures of the comparisons between the groups are shown in Figures 1 and 2. T-student, odd ratios, chi-square and/or Fisher's exact tests were used to evaluate differences, with z-tests applied to compare column proportions. Also, p-values were adjusted using the Bonferroni and Benjamini-Hochberg (FDR) statistical methods. An adjusted-p-value < 0.05 was considered indicative of a statistically significant difference.

RESULTS

MS-MLPA.

DNA of 101 BWSp patients with previously confirmed methylation defects at the 11p15.5 (MS-MLPA-ME030) locus were re-investigated using the MS-MLPA-ME034 kit revealing the presence of MLID in 16 of the patients (15.8 %) (Table S1). As expected, all these patients identified with MLID exhibited LoM at ICR2, while none of the patients with isolated GoM at ICR1 or with UPD showed MLID. Abnormal methylation patterns were observed exclusively at maternally imprinted loci, specifically *PLAGL1* (6q24.2), *GRB10* (7p12.1), *MEST* (7q32.2), *SNRPN* (15q11.2), *GNAS-NESP55* (20q13.32), *PEG3* (19q13.43), and *GNAS* (20q13.32). Among these, *MEST* and *GNAS* were the most frequently affected loci, each altered in 43.8 % of MLID-BWSp patients (Figure 3A). None of the patients showed abnormal methylation of the *MEG3*, *MEG8* and *H19* loci. Eleven patients had LoM at only one additional locus, three had LoM at two additional loci, one patient showed LoM at five additional loci and one patient exhibited LoM at two additional loci and GoM at one (Table S1).

Methylation microarrays.

All the results obtained by MS-MLPA-ME034 were also confirmed with the methylation microarray, with an exception. In contrast to the MS-MLPA-ME034 results, no abnormal methylation was detected at *MEST* for patient #87. Additionally, patients #23, #31, #40, #56, #69, #86, #89 and #101, had LoM in the ICR2 region detected by both chromosome 11 specific MS-MLPA-ME030 and MS-MLPA-ME034, but was not identified with the methylation microarray. In individuals #23, #31, #56, #86, #89 and #101, the hypomethylation level did not exceed -3 SD but was detected at -2 SD.

Notably, by studying the CA-DMRs described by Court and colleagues (28), we identified 43 additional patients with MLID, increasing the occurrence of MLID to 59 out of 101 BWSp patients (58.4 %); the distribution of the complete BWSp cohort is illustrated in Figures 1 and 2. We detected abnormal methylation status in 28 DMRs of which eight corresponded to regions previously identified as abnormally methylated using MS-MLPA-ME034: *KCNQ1OT1*, *GNAS*, *GNAS-NESP55*, *MEST*, *GRB10*, *PLAGL1*, *SNRPN* and *PEG3*. Notably, the methylation microarray also revealed abnormal methylation in some of these CA-DMRs in patients who were not detected by MS-MLPA-ME034. The remaining 20 DMRs were not covered by the MS-MLPA-ME034 and include: *DIRAS3* (1p31.3), *FAM50B* (6p25.2), *ZDBF2* (2q33.3), *L3MBTL1* (20q13.12), *NAP1L5* (4q22.1), *SNU13* (22q13.2), *GET1* (21q22.2), MEG3 (14q32.2), GPR1 (2q33.3), MCTS2P (20q11.21), PEG13 (8q24.3), *ERLIN2* (8p11.23), *MKRN3* (15q11.22), *NNAT* (20q11.23), *PPIEL* (1p34.3), *RB1* (13q14.2), *ZNF331* (19q13.42), *ZNF597* (16p13.3), *HTR5A* (7q36.2) and *PEG10* (7q21.3). As shown in Figure 3B, other regions, such as *DIRAS3*, *FAM50B* and *L3MBTL1*, emerged as having abnormal methylation at similar frequencies than *GNAS* or *MEST*. Table S2 and Figures S1 and S2 show detailed information about the methylation status of these patients across the identified DMRs with abnormal methylation status.

To further investigate additional epigenetic alterations beyond the CA-DMRs, we analysed regions with abnormal methylation patterns compared to controls, excluding the previously assessed CA-DMRs. This analysis revealed that 81 out of the 101 BWSp patients exhibited aberrant methylation in non-imprinted regions (Figures 2 and S2). Of these 81 patients, 56 had been previously classified as MLID-BWSp, while the remaining 25 were BWSp patients without methylation alterations in the assessed CA-DMRs. The differentially methylated non-imprinted regions included *HR*, *JAKMIP1*, *HOXB6*, *MIRLET7BHG*, *TRAJ4*, *ZNF503*, *BST2*, *GNG12*, *OTAIRM1*, *SNED1*, *PRRT1*, *TRAJ39*, *STRA6*, *APOB*, *GCNT2*, *EXD3* and *TROAP*, most of which were hypomethylated, except for *BST2* which showed hypermethylation. Interestingly, *JAKMIP1*, *HOXB6* and *SNED1* have been reported to be candidate imprinted DMRs in genome-wide methylation screens using reciprocal maternal and paternal uniparental diploidy samples (31).

Statistical analysis of clinical features

The clinical features of the BWSp cohort are summarized in Table 1 (59 MLID-BWSp) and Table 2 (42 non-MLID BWSp). Cardinal, suggestive and other clinical features' frequencies, OR, pvalues and interpretations of each analysis are described in Table S3. We subdivided the entire cohort of 101 BWSp patients in three groups: group A: MLID-BWSp diagnosed by MS-MLPA-ME034, group B: MLID-BWSp diagnosed by microarray and group C: Non-MLID-BWSp (Figure 1). We have not observed statistical differences between groups A, B and C regarding the clinical features. Additionally, pairwise comparison was then performed (Table S3). Results did not show statistically significant differences between groups A, B and C (Figures 1 and 2). In the subsequent analysis, comparing 81 BWSp patients with DMRs outside imprinted regions to 20 patients without DMRs outside imprinted regions, no clinical features were found to be significantly more frequent in either group (Figure 2, Table S3 panel C). A third analysis, comparing 56 MLID-BWSp patients with DMRs in non-imprinted regions and 25 non-MLID BWSp patients with DMRs in non-imprinted regions, revealed no statistical differences as well. Seven out of the 48 MLID-BWSp patients with complete data on type of conception (14.6 %) and 3 out of 42 (7,1%) non-MLID BWS group identified in this study were conceived through ART. This difference did not reach statistical significance but a tendency (Fisher's exact test; OR: 2.22; p 0.32). At molecular level, we have observed significant differences in the mean number of aberrant DMRs between MLID-BWSp cases initially detected by MLPA (group A) and those detected exclusively by microarrays (group B) (pvalue = 0.0033, Student's t-test). The mean number of DMRs was approximately 1.9-fold higher in patients identified by MLPA compared with those detected solely by microarrays (Table 3). These findings were consistent in both classical and atypical BWSp, suggesting that although the extent of methylation involvement (i.e., number of DMRs) varies depending on the detection method, the overall clinical presentation and distribution remain comparable.

Discussion

In this study, we investigated the DNA methylation profiles of 101 patients with BWSp using MS-MLPA-ME034 and methylation microarrays to characterize the presence of MLID and assess potential epigenetic alterations beyond the CA-DMRs (28). MLID was identified in 16 patients (15.8 %) using MS-MLPA-ME034 (11 loci), and in 59 patients (58.4 %) by methylation microarrays. These figures align with previous reports, where MLID frequencies in BWSp range from 10% to 50%, depending on the methodology. Fontana et al. (8) reported 50% of MLID using a similar platform; Bliek et al. (12) and Urakawa et al. (15) found 20% and 12%, respectively. This variability reflects the impact of the analytical method. High-density microarrays allow broader genomic coverage and capture subtle changes missed by MS-MLPA-ME034. This was also observed by Kim et al. (22) who showed that microarrays detected MLID patients overlooked

by MS-MLPA-ME034. Our findings highlight the value of incorporating genome-wide methylation approaches, such as methylation microarrays, into diagnostic workflows to improve the detection and clinical management of MLID-BWSp, while MS-MLPA-ME034 remains a practical tool for routine cases. In contrast, pyrosequencing, now seldom used in epigenetic diagnostics, limits analysis to individual loci within single assays and is therefore not recommended for evaluating MLID or detecting methylation across multiple loci.

All MLID patients exhibited ICR2 LoM, consistent with previous studies identifying this as a hallmark lesion in MLID-BWSp. ICR2 regulates *CDKN1C*, a gene critical for growth regulation. LoM leads to *CDKN1C* silencing, contributing to the overgrowth seen in BWSp patients. The *International Consensus* on BWSp diagnosis and management recognizes ICR2 LoM as a key diagnostic feature (3,11). Bliek et al. (12) also found universal ICR2 LoM in their MLID cohort. This consistency across studies underlines the importance of ICR2 LoM as a diagnostic marker and a target for further research into therapeutic interventions aimed at restoring normal methylation patterns, such as targeted epigenetic therapies, which are currently under investigation in other imprinting disorders (32–34).

We also observed recurrent LoM at *GNAS*, *MEST*, *GRB10*, and *PLAGL1*, consistent with previous studies (8,12,16,21). These loci are involved in growth and metabolism, and their dysregulation likely exacerbates BWSp traits. For instance, *GNAS* encodes the alpha subunit of a stimulatory G protein; its LoM can impair multiple signalling pathways (21). *MEST*, a maternally imprinted gene critical for foetal growth, also shows LoM in MLID (35). Methylation microarrays detected additional loci not interrogated by the MS-MLPA-ME034, such as *DIRAS3*, *FAM50B*, and *ZDBF2*. The frequency of aberrant methylation at these loci was comparable to, or exceeded, that observed in the CA-DMRs, highlighting their potential relevance for future inclusion in MS-MLPA-ME034 panels. *DIRAS3* is a maternally imprinted tumour suppressor gene expressed from the paternal allele. Its expression is regulated through CpG island methylation, and the loss of *DIRAS3 activity* can affect *PI3K/AKT* and *RAS/MAPK* pathways (36). Although no statistically significant clinical differences were found between patients with and without *DIRAS3* methylation abnormalities (data not shown), we observed a trend toward a higher prevalence of hemihyperplasia and macrosomia among those with aberration of *DIRAS3*. This pattern may suggest a potential phenotypic association that merits further investigation in larger cohorts.

FAM50B, a paternally expressed gene on chromosome 6, has an unclear function but is increasingly associated with MLID. Previous reports (37,38) identified *FAM50B* LoM in MLID, including in BWSp (22), implying a role in the syndrome's complex epigenetics. Further research is needed to define its function and potential diagnostic or therapeutic relevance. *ZDBF2*, located on 2q33.3, is a paternally imprinted gene whose function is poorly understood. In mice, it regulates neonatal growth in correlation with *IGF-1* levels (39). We detected GoM at *ZDBF2* in

10 of 59 MLID-BWSp patients, likely secondary to LoM of the *CMKLR2-AS* (*GPR1-AS*) DMR (40), which controls its expression.

L3MBTL1 is a Polycomb-group chromatin regulator that binds mono- and dimethylated lysines to promote chromatin compaction and transcriptional repression. In humans, it is paternally expressed due to monoallelic CpG methylation, a species-specific imprinting pattern absent in mice. Deletion or reduced expression of *L3MBTL1* in the 20q12 region contributes to genomic instability and myeloid malignancies, supporting its role as a tumour suppressor.

Beyond CA-DMRs, our study found methylation changes at other loci including *HOXB6*, *MIRLET7BHG*, and *APOB*, genes involved in development and metabolism (41-43). Most showed hypomethylation, but *BST2* was hypermethylated, pointing to distinct regulatory mechanisms. While their clinical impact is unclear, they may contribute to BWSp variability and deserve further investigation. We also observed more pronounced hypomethylation in patients with BWSp scores ≥4 at loci like *PLAGL1* and *L3MBTL1*, and to a lesser degree at non-imprinted sites like *JAKMIP1* and *GNG12*, compared to those with scores ≤3 (Figures S1 and S2).

Table S3 provides a comparative analysis of clinical features across the three BWSp subgroups (MLID-BWSp microarray, MLID-BWSp MLPA, and non-MLID BWSp), highlighting that most phenotypic findings occur at similar frequencies across groups. These results support the notion that molecular heterogeneity within BWSp (including MLID status and detection method) has limited impact on the overall phenotypic spectrum. This reinforces previous observations that BWSp remains primarily defined by the imprinting defect at 11p15, with MLID acting as an additional molecular finding rather than a major clinical modifier.

There were no sex-based differences in MLID prevalence (23 females, 22 males). As mentioned above, clinical features differed between MLID-BWSp and non-MLID-BWSp but without statistical differences. Macroglossia, transient hypoglycemia, nevus and hypotonia tend to be more frequent in the MLID-BWSp group. At the molecular level, patients identified by MLPA exhibited a mean number of DMRs approximately 1.9-fold higher than those detected solely by microarrays. These findings underscore the complementary roles of MS-MLPA-ME034 and genome-wide methylation microarrays in MLID diagnostics: while MS-MLPA-ME034 remains suitable for a rapid, routine screening, the integration of high-resolution methylation microarrays into diagnostic pipelines enhances detection sensitivity (more DMRs) and may support the clinical delineation of complex or atypical patients.

There is increasing evidence that ART may raise the risk of imprinting disorders, including BWSp, due to methylation instability. Previous studies, including our own (44), found higher BWSp prevalence in ART-conceived individuals. In the present cohort, pregnancies conceived through ART were more frequent among individuals with MLID than among non-MLID cases (OR = 2.22); however, this difference did not reach statistical significance (Fisher's exact test, $p = 0.32$), likely due to the limited sample size and the low absolute number of ART

conception. These findings support incorporating epigenetic screening into prenatal care for ART pregnancies, as Mussa et al. recommend (4), because early detection may improve outcomes. Optimizing embryo handling and reducing in vitro manipulation could help lower imprinting disorder risks (45).

Limitations of this study are the relatively small number of patients with BWSp and the fact that we were unable to test maternal DNAs. Although identifying maternal-effect variants would help to clarify MLID mechanisms and recurrence risk, most maternal samples were unavailable due to the retrospective study design. Thus, we could not assess variants in *SCMC*-related genes. This limitation highlights the need for future studies with parental samples.

In summary, this study highlights the complexity of MLID in BWSp. We identified MLID in 58.4 % of patients through combined MS-MLPA-ME034 and microarray analysis. Recurrent involvement of loci such as *GNAS*, *MEST*, *FAM50B*, *DIRAS3*, *L3MBTL1*, and *ZDBF2*, along with others like *BST2*, *GNG12*, and *APOB*, underscores the broader epigenetic disruptions in BWSp. These findings support a stratified diagnostic approach to MLID, where MS-MLPA-ME034 remains appropriate for initial clinical screening, while the integration of genome-wide methylation microarrays enhances diagnostic resolution and might help clinical management, particularly in patients with atypical phenotypes or suspected complex epigenetic alterations. Future studies should assess long-term outcomes of MLID and its molecular underpinnings. This study also provides new insights into the epigenetic landscape of MLID in BWSp, emphasizing the consistent presence of ICR2 LoM, variability in MLID prevalence, and the range of epigenetic and clinical diversity in affected individuals.

Author contributions

Alejandro Parra: data collection, investigation, conceptualization, writing original draft and review. Mario Cazalla: investigation, conceptualization, software, writing original draft and review. Carlos Rodríguez-Antolín: software and validation. Cristina Silván, Lucía Miranda-Alcaraz, Mónica Mora-Gómez, Natalia Gallego-Zazo, Manuel Rodríguez-Canó, Juan A. Jiménez-Estrada and Pedro Arias: investigation and data collection. Enrique Galán Gomez, Antonio González-Meneses, Pablo Barbero, Vanesa Lotersztein, Spanish OverGrowth Registry Initiative (SOGRI), Alfredo Santana: resources and data collection. Jair Tenorio-Castano: conceptualization, investigation, review, supervision and funding acquisition. Julián Nevado: conceptualization, investigation and review. Mathis Hildonen, Zeynep Tümer, Victor L Ruiz-Perez, David Monk: investigation and review. Pablo Lapunzina: conceptualization, investigation, review, supervision and funding acquisition. All the authors commented to the manuscript and accepted the final version.

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DECLARATIONS

Ethical Approval

This study was approved by the Ethical Committee of the Hospital Universitario La Paz (CEIm PI-446), and

Consent to Publish declaration: Informed consent was obtained from all patients and/or their parents/ legal guardians.

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Availability of data and materials

Not applicable

Conflict of interest statement

The authors declare no conflicts of interest.

Author contribution: MC and AP contributed writing the manuscript, MC and CRA performed bioinformatic methylation analysis, CS, LMA, MMG, NGZ, PA, MRC contributed to investigation and data collection, JJE contributed with figure preparation and molecular studies, EGG, AGM, PB, VL, SOGRI, FR and AS contributed to acquisition of resources and patient data collection. JTC, JN, contributed to conceptualization, investigation, critical manuscript review, supervision, and

funding acquisition. MH and ZT contributed with data analysis support and critical review of the manuscript. VLRP and DM contributed to investigation and manuscript review. PL served as senior investigator, contributing to conceptualization, investigation, critical manuscript review, supervision, and funding acquisition. All authors reviewed and approved the final manuscript version prior to submission.

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Legends for Figures

Figure 1. Distribution of BWSp according to the MLID status. A) BWSp-MLID patients diagnosed by MS-MLPA-ME034, B) BWSp-MLID patients diagnosed only by microarray, C) non-MLID-BWSp patients. A microarray analysis was finally performed in all 59 patients with MLID-BWSp.

Figure 2. Distribution of BWSp patients according to both the methylation status (DMRs) and MLID status in non-imprinted regions. DMR refers here to abnormally methylated regions (e.g., hypomethylated or hypemthylated loci) detected in patients.

Figure 3. A. Frequency of methylation aberrations observed across the 11 imprinted loci analysed by MS-MLPA-ME034. Blue bars indicate hypomethylation, and the red bars indicate hypermethylation. The chromosomal locations of these loci are listed in Table S1. GOM: gain of methylation; LOM: loss of methylation. [1], [2] and [3] correspond to different probes in the same gene from the MS-MLPA-ME034 assay. **B.** Frequency of methylation aberrations across the differentially methylated regions (DMRs) identified using the methylation microarray. Blue bars represent hypomethylation, and red bars represent hypermethylation.

Figure S1. Methylation status of BWSp patients and controls across the maternally imprinted loci. DNA methylation β-values are represented on a color scale ranging from blue (low methylation) to red (high methylation). The heatmap is first ordered by the Brioude Score (3) of each patient (blue for patients with a score ≤ 3; red for patients with a score ≥ 4; gray for controls). Within these groups, samples are further ordered by MLID status (yellow for non-MLID BWSp; red for MLID-BWSp; gray for controls). The heatmap was generated using the ComplexHeatmap package in the R software environment.

Figure S2. Methylation status of BWSp patients and controls across the non-imprinted regions. DNA methylation β-values are represented on a color scale ranging from blue (low methylation)

to red (high methylation). The heatmap is first ordered by the Brioude Score (3) of each patient (blue for patients with a score ≤ 3; red for patients with a score ≥ 4; gray for controls). Within these groups, samples are further ordered by MLID status (yellow for non-MLID BWSp; red for MLID-BWSp; gray for controls). The heatmap was generated using the ComplexHeatmap package in the R software environment.