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## Review

Deborah J.G. Mackay, Thomas Eggermann, Karin Buiting, Intza Garin, Irène Netchine, Agnès Linglart and Guiomar Perez de Nanclares\*

# Multilocus methylation defects in imprinting disorders

**Abstract:** Mammals inherit two complete sets of chromosomes, one from the father and one from the mother, and most autosomal genes are expressed from both maternal and paternal alleles. In imprinted genes, the expression of the allele is dependent upon its parental origin. Appropriate regulation of imprinted genes is important for normal development, with several genetic diseases associated with imprinting defects. A common process for controlling gene activity is methylation. The first steps for understanding the functions of DNA methylation and its regulation in mammalian development have led us to identify common (epi)genetic mechanisms involved in the eight human congenital imprinting disorders.

**Keywords:** Angelman syndrome; Beckwith-Wiedemann syndrome; imprinting disorder; multilocus methylation defect; Prader-Willis syndrome; pseudohypoparathyroidism

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type 1B; Silver-Russell syndrome; Temple syndrome; transient neonatal diabetes; Wang-Kagami-Ogata syndrome.

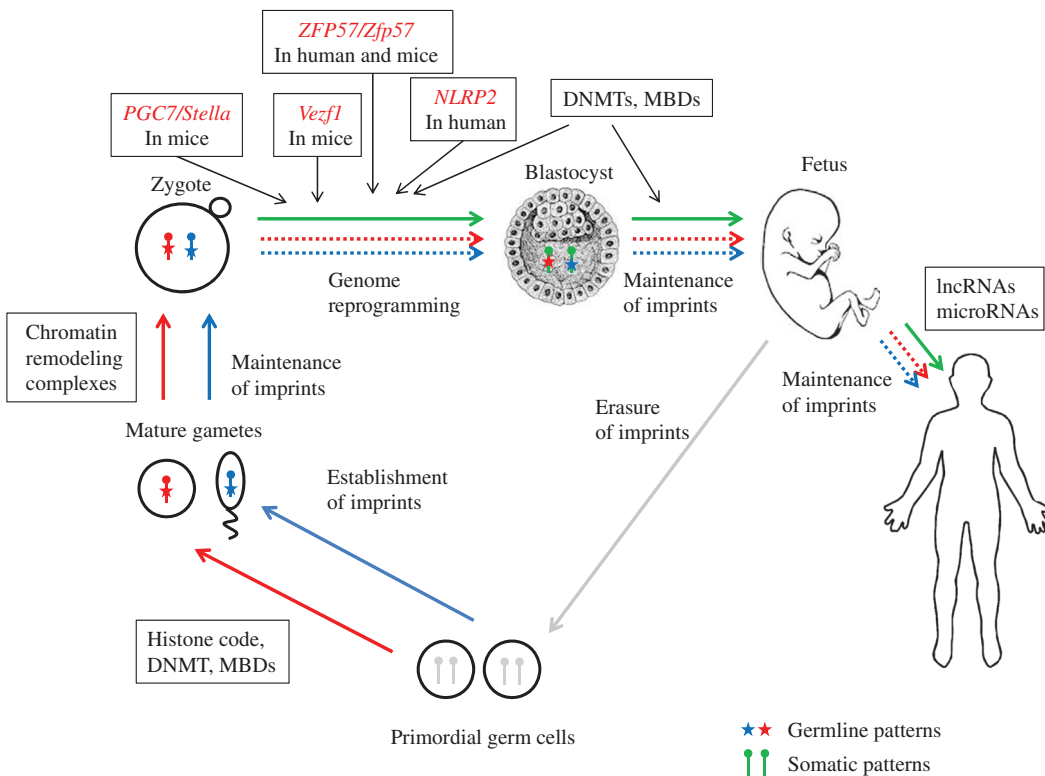
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## Introduction

The term “genomic imprinting” refers to the expression of specific genes in a parent-of-origin specific manner, that is, expression from the maternal or the paternal gene copy only (1). It is controlled by epigenetic modifications, including DNA methylation within differentially methylated regions (DMRs). To date, more than 70 human genes have been classified as imprinted, with many more likely awaiting identification (<http://igc.otago.ac.nz>) (2, 3), though the exact number is not clear (3). The normal imprinting marks are inherited from the parental gametes and are maintained in the somatic cells of an individual (Figure 1). However, this mechanism is prone to various disturbances resulting in distinctive pathological courses, including malignant tumours or – in cases of parentally imprinted genes – imprinting disorders (IDs) (4, 5).

IDs are a group of congenital diseases affecting growth, puberty, behavior, development, and metabolism, with lifelong impacts on patients' quality of life (2). In nearly all known IDs, the same classes of molecular changes are detectable: (i) uniparental disomy (UPD), the inheritance of both chromosomal homologs from the same parent; (ii) deletions/duplications; (iii) epimutations, aberrant methylation without alteration of the genomic DNA sequence; and (iv) point mutations in imprinted genes (6). At present, there are eight clinically well-recognized IDs (Table 1): the Beckwith-Wiedemann syndrome (BWS, OMIM 130650), Silver-Russell syndrome (SRS, OMIM 180860), Prader-Willi syndrome (PWS, OMIM 176270), Angelman syndrome (AS, OMIM 105830), Temple



**Figure 1** Illustration of the epigenetic reprogramming cycle and the known factors involved in its regulation.

In the fetal germline, all DNA methylation patterns are erased (gray line), and then paternal (blue) and maternal (red) methylation imprints are established during gametogenesis. The two germline genomes that are combined at fertilization undergo parent-specific genome reprogramming in the early embryo, during which most germline patterns are erased again and somatic patterns (green) are established. Only imprinted genes maintain their germline patterns during development of the new organism. DNMT, *de novo* methyl transferase; MBDs, methyl binding domain proteins; IncRNAs, long non-coding RNAs. Adapted from Reik and Walter (1) and Azzi et al. (51).

**Table 1** Overview on the known congenital imprinting disorders in humans.

|                               | AS           | PWS              | BWS                | SRS         | mUPD14-like      | pUPD14-like      | TNDM          | PHP1B                           |
|-------------------------------|--------------|------------------|--------------------|-------------|------------------|------------------|---------------|---------------------------------|
| Frequency                     | 1/16,000     | 1/18,000         | 1/13,000           | 1/70,000    | Unknown          | Unknown          | 1/500,000     | Unknown                         |
| Chromosome                    | 15q11–13     | 15q11–13         | 11p15              | 11p15/7p12  | 14q32            | 14q32            | 6q24          | 20q13                           |
| Candidate gene                | <i>UBE3A</i> | <i>SNORD116</i>  | <i>IGF2/CDKN1C</i> | <i>IGF2</i> | <i>GTL2/DIO3</i> | <i>GTL2/DIO3</i> | <i>PLAGL1</i> | <i>GNAS</i>                     |
| Cytogenetic rearrangement (%) | 75           | 75               | 5                  | 5           | Rare deletions   | Rare deletions   | 29            | Deletion in <i>STX16/NESPas</i> |
| DNA mutation (%)              | 10           | <1               | 5                  | Once        | 0                | 0                | 0             | Familial cases                  |
| UPD (%)                       | 5            | 25               | 20                 | 10          | 50 reported      | 30 reported      | 41            | 6 reported                      |
| Epimutation (%)               | 5            | <1               | 55                 | 35–50       | 3 reported       | 1 reported       | 30            | Sporadic cases                  |
| MLMD                          | MLMD once    | Not yet reported | MLMD               | MLMD        | Not yet reported | Not yet reported | MLMD          | MLMD                            |

MLMD, multilocus methylation defect.

syndrome (7), Wang-Kagami-Ogata syndrome (OMIM 608149), transient neonatal diabetes (TNDM, OMIM 601410), and pseudohypoparathyroidism type 1B (PHP1B, OMIM 603233), though this number is increasing (8). The clinical spectrum of IDs is broad, some features are subtle, and thus some IDs are likely underdiagnosed.

Initially, each of these disorders was described separately, and molecular defects in specific chromosomal regions were identified for each; however, over the last 5 years, several clinical and molecular overlaps among the different IDs have become obvious. In particular, many patients with different clinically diagnosed IDs have been

reported to carry epigenetic mutations not only at the disease-specific loci but also in other chromosomal regions (9–15). The identification of these so called “multilocus methylation defects” (MLMDs) illustrates that IDs should be considered not separately but as a single entity, given their likely similar pathogenic mechanisms (6).

The first evidence of MLMDs was reported by Arima et al. (15) and Mackay et al. (12), who identified patients with TNDM carrying a *PLAGL1/ZAC1* epimutation and a hypomethylation of imprinting control region (ICR) 2 in 11p15 (also known as KvDMR), the most frequent BWS epimutation. Recently, mutations in the *ZFP57* gene have been described in some of these patients with TNDM and an MLMD, making this the first gene linked to an autosomal recessively inherited ID (16).

Subsequently, MLMDs have been detected in other IDs, with both paternally and maternally imprinted loci affected. The highest reported frequency is in TNDM (50% of epimutation carriers), followed by BWS, detectable in 25% of carriers of an ICR2/KvDMR hypomethylation in 11p15 (9, 10, 14). Given that this epimutation accounts for 50% of patients (17), it could be deduced that ≈12% of patients with BWS are MLMD carriers. MLMDs are less frequent in SRS (9, 18) and PHP1B (13, 19, 20), detected in 8%–10% of epimutation carriers. MLMDs have been described in other IDs only rarely. However, MLMD can differ for the same patient when screened in different tissues and different tissue-specific MLMDs co-existed in 38% of Silver-Russell Syndrome (SRS) patients with 11p15 loss of methylation (LOM) in leukocytes. These tissue-specific epigenotypes may contribute to the clinical heterogeneity observed in IDs (21).

We will summarize the specific (epi)genetic characteristics of the different IDs as well as the effect of the MLMD in some of them.

## Chromosome 6q24: TNDM

The 6q24-related transient neonatal diabetes mellitus (6q24-TNDM) is characterized by diabetes mellitus that presents within the first 6 weeks of life in a term infant and resolves by age 18 months (22, 23). It is caused by overexpression of the imprinted genes at 6q24 (*PLAGL1/ZAC*) and *HYMAI* (24, 25). Normally, expression of the maternal alleles of *PLAGL1* and *HYMAI* are silenced by DMR methylation, and only the paternal alleles of *PLAGL1* and *HYMAI* are expressed (26). Three different genetic mechanisms result in twice the normal dosage of these two genes and cause 6q24-TNDM: paternal UPD of

chromosome 6 [UPD(6)pat, 41% of cases]; duplication of 6q24 on the paternal allele (29%); and hypomethylation of the maternal DMR resulting in inappropriate expression of the maternal *PLAGL1* and *HYMAI* alleles (30%) (27–30). This hypomethylation is invariably complete, which may reflect the ascertainment bias of clinical presentation.

Over half of patients with maternal hypomethylation at the TNDM locus have additional hypomethylation of other maternally methylated imprinted genes throughout the genome (30).

Homozygous or compound heterozygous *ZFP57* mutations account for almost half of TNDM-MLMD; the other causes of MLMD are not known (16). The finding of mosaic epigenetic aberrations associated with *ZFP57* mutations indicates that the gene may be involved in maintenance of DNA methylation at imprinted regions during the early multicellular stages of human development, similarly to its role in the mouse (31). The epigenetic signature of *ZFP57* homozygous and compound heterozygous individuals includes total LOM of *PLAGL1* DMR, partial hypomethylation of *PEG3* DMR (19q13.4), and partial or complete hypomethylation of *GRB10* DMR (7p12.2) (16, 32). In other patients with TNDM-MLMD, no underlying causative mutation has yet been identified. Among these patients, the affected loci identified so far are maternally methylated and may include *MEST/PEG1* (7q32), ICR2/KvDMR (11p15) and *GNAS* (20q13) (12, 30, 33).

Regarding the influence of the multilocus defect over the phenotype, non-diabetes manifestations are more likely in the subgroup with generalized hypomethylation at imprinted loci and can include significant learning difficulties, marked hypotonia, congenital heart disease, deafness, neurologic features including epilepsy, and renal malformations (11, 16). No correlation has been observed between clinical severity and either the degree of hypomethylation or the range of loci involved. For example, the features seen in individuals with 6q24-TNDM caused by homozygous or compound heterozygous *ZFP57* mutations can vary from severe intellectual disability and early infant death to a normal phenotype (32).

## Chromosome 11p15: BWS/SRS

BWS is a growth disorder characterized by macrosomia, macroglossia, visceromegaly, omphalocele, neonatal hypoglycemia, ear creases/pits, adrenocortical cytomegaly, increased risk to embryonal tumors (e.g., Wilms tumor, hepatoblastoma, neuroblastoma, and rhabdomyosarcoma), and renal abnormalities (e.g., medullary dysplasia,

nephrocalcinosis, medullary sponge kidney, and nephromegaly) (17). BWS is caused by molecular alterations in the chromosomal region 11p15.5 which harbors two imprinting control regions (ICR1/*H19* and ICR2/*KvDMR*). Deregulation of genes controlled by either the ICR1 or ICR2 results in the BWS phenotype through the change of the relative contributions of parental alleles (34). Five major different disturbances in 11p15.5 have been described to be associated with BWS: (i) Sporadic LOM at the ICR2 occurs in 50% of patients. (ii) Gain-of-methylation (GOM) at the ICR1 is detectable in 5%; some of these methylation alterations have been associated with genomic alterations (35), and these methylation changes are important because of their heritability. Up to 20% of the GOM at the ICR1 are secondary to genetic anomaly within the ICR1 affecting CTCF and OCT4/*SOX2* binding sites (36). (iii) Segmental paternal UPD(11)pat of the region 11p15.5 is diagnosed in 20% of BWS patients. Segmental UPD often arises from a post-zygotic somatic recombination event and therefore has a mosaic distribution (17). (iv) A minor fraction of BWS patients carry duplications/deletions of paternal chromosome 11p15.5 material. (v) In up to 5% of BWS patients, point mutations within the *CDKN1C* gene in 11p15.5 affecting the maternal allele can be detected. It is now clear that the risk of tumor occurrence differs considerably between the various underlying molecular defects with a higher incidence of tumor when the ICR1 domain is involved (37).

The same chromosomal region 11p15.5 is altered in patients with SRS, a congenital disorder characterized by severe prenatal and postnatal growth retardation, relative macrocephaly at birth, a distinctive triangular face with prominent forehead, low-set ears, clinodactyly of the fifth fingers, feeding difficulties, and body asymmetry (38, 39). Genetic and epigenetic disturbances can be detected in approximately 50% of patients with typical SRS features (40), and the majority of patients show a chromosome 11p15.5 disturbance. Hypomethylation of the paternal imprinting center 1 (IC1) of chromosome 11p15.5 is identified in 35%–50% of individuals with SRS (41–43). A small number of individuals with SRS have a duplication involving the maternal 11p15.5 region (44). Recently, an activating mutation of *CDKN1C* has been described in a familial case of SRS, completing thereby the molecular mirror of SRS causes with the molecular causes identified in BWS (45).

As mentioned before, MLMD in BWS is found in 25% (46) of patients with ICR2 hypomethylation (9, 10, 14, 47) and in 10% of SRS with hypomethylation of *H19* ICR1-DMR (11p15.5) (9, 18). In a very recent paper, MLMD was found in two out of 10 BWS patients with ICR1/*H19* hypermethylation (48). In some of these patients, the hypomethylation can involve paternally or maternally methylated DMRs (9),

in contrast to *ZFP57*-related TNDM MLMD patients in whom the hypomethylation occurs only at maternally imprinted loci. However, no underlying genetic defect for BWS or SRS MLMD cases is so far known, except for a single case with a homozygous sequence mutation in the *NLRP2* gene in the mother of two siblings with BWS and hypomethylation of the ICR2 and MLMD in one of the twins (49).

Although the analyzed loci and techniques are not consistent in the different studies on MLMD, preliminary data show that the affected loci vary between groups of patients. Whereas *PLAGL1*, *GRB10*, and *MEST/PEG1* are affected both in BWS-MLMD and SRS-MLMD (6), the predominantly involved loci apart from ICR2 in BWS patients are *GNAS* and *IGF2R* (9, 10, 14, 47), whereas in SRS, *DLK1* is often affected (9).

In many MLMD patients ascertained with a BWS or SRS phenotype (9, 14), no additional clinical features compared with those of single locus hypomethylation BWS patients were noted. For example, there was no clinical history of TNDM in the BWS (10) or SRS (9) patients who had total or partial LOM of *PLAGL1* DMR. Moreover, MLMD patients with the same aberrant methylation patterns in lymphocytes but either a BWS or a SRS phenotype have been identified (9). However, some SRS MLMD carriers present with a severe clinical spectrum, including developmental delay and dysmorphism (9, 18). Furthermore, among three of 77 SRS patients with hypomethylation at both ICR1 and ICR2, one had an umbilical hernia that is not typical for SRS but a common feature for BWS (50). In some patients with MLMDs with a BWS phenotype, both birth weight and frequency of macrosomia are lower than in other BWS subgroups, and features not typically seen in BWS (e.g., speech retardation, apnea, feeding difficulties) are present (10, 47). To explain the phenotypic outcome in MLMD carriers, Azzi et al. suggested that the most severe epimutation, i.e., the lowest methylation level, might be clinically dominant, and in cases with comparable levels of hypomethylation at multiple loci, one locus may have an (epi)dominant effect over the other(s) (9, 51). Nevertheless, we have to keep in mind that the studies published are based on methylation analyses at lymphocyte DNA, and it is possible that the mosaic distribution in other tissues would influence the phenotypic expression. Furthermore, only a limited number of imprinted loci have been tested.

## Chromosome 7: SRS

About 10% of individuals with SRS have maternal UPD for chromosome 7 (40, 52). Clinically, it should be considered

that UPD(7)mat patients generally show a slightly different phenotype in comparison to 11p15 hypomethylation carriers (53, 54): while growth is less restricted and asymmetry is less frequent in UPD(7)mat carriers, these patients are more likely to have delayed development and speech (54).

Two different candidate regions have been proposed: 7p11.2–p13 and 7q32. For the candidate region in 7p11.2–p13, SRS patients with duplications have been reported (55, 56). The region harbours an imprinted gene (growth factor receptor bound protein 10/*GRB10*) and several factors involved in human growth and development (*IGFBP1*, *IGFBP3*, *PHKG1*, *EGFR*, and *GHRHR*). Pathogenic mutations in these genes have been excluded in SRS (57). In particular, *GRB10* plays an essential role in growth and is therefore still a good candidate for SRS. This assumption was supported by a published family carrying a maternally inherited dup(7)(p11.2p12) not including *GRB10* and without SRS features (58). However, several studies argued against a major role of *GRB10* mutations in the etiology of SRS (59), and it became obvious that an aberrant imprinting of *GRB10* in 7p12 does not significantly contribute to the SRS phenotype as several patients with segmental maternal UPD restricted to the long arm of chromosome 7 (UPD(7q)mat) have been identified (60). The only SRS patient with methylation defect at *GRB10* also carried a 20p13 microdeletion, so it is unclear whether the phenotype is attributable to the *GRB10* hypermethylation or to the 20p13 deletion (61).

Three imprinted genes (*MEST/PEG1*, *CPA4*, and *COPG2*) and two imprinted non-coding RNAs (*MESTIT1* and *CIT1/COPG2IT1*) are located in 7q32. In particular, the *MEST/PEG1* gene was regarded as a convincing candidate gene for SRS since *Peg1/Mest* knockout mice show prenatal and postnatal growth failure when the mutant gene is transmitted from the father (62). Both *MEST/PEG1* and *MESTIT1* have been discussed as candidate gene for SRS, but screening studies for point mutations as well as methylation studies have not detect any pathogenic variants or aberrant methylation patterns so far (63–66), with the exception of a case with a *de novo* deletion in 7q32 affecting the paternal imprinted *MEST/PEG1* gene copy (67).

Schönherr et al. showed that further epigenetic defects did not occur in the groups of SRS with UPD(7)mat and that this entity does not belong to the diseases with a general hypomethylation defect (68). However, MLMD is generally not detectable in UPD cases. The patient with isolated hypermethylation at *GRB10* was also investigated for MLMD, with negative results (61).

However, further studies are needed to identify the gene conclusively implicated in SRS. A very recent report of Hannula-Jouppi and colleagues has suggested many new imprinted sites that need to be tested in these patients (69).

## Chromosome 20q13: PHP1B

PHP1B is a rare disease characterized by kidney resistance to parathyroid hormone (PTH) and, in some cases, mild resistance to thyroid-stimulating hormone. In a few patients, features of Albright hereditary osteodystrophy (short stature, ectopic ossifications, and bone shape abnormalities) and/or obesity may be present (70).

About 15%–20% of patients with PHP1B display a LOM restricted to the A/B DMR, associated with maternal 3-kb micro/4.4-kb deletions within the *STX16* gene (71, 72). These patients are described here as having autosomal dominant PHP1B (AD-PHP1B). A very small number of families with PHP1B have been reported to have a broad methylation defect at the *GNAS* locus (LOM at AS/NESPAS, XLas, and exon A/B and GOM at NESP55) and a deletion removing *AS* exons 3 and 4 (73, 74), a deletion removing *NESP55* and *AS* intron 4 (75) or very small deletions within *NESP55* (76). Most patients with PHP1B (80%–85%) have a sporadic disease, with broad methylation defect encompassing the *GNAS* locus, without microdeletions within the *STX16* or *AS* genes (77, 78) and in a few cases associated with UPD(20q)pat (79–81).

MLMD is an infrequent event in PHP1B patients (13) that includes both hypermethylation and hypomethylation at maternally and paternally imprinted loci, without any specific grouping (19, 20). Regarding the influence of the other methylation alterations on the phenotype of these MLMD-PHP1B patients, there is no significant correlation between the imprinting defect and PTH resistance or weight/height parameters (13, 20). The only atypical features found were hypercholesterolemia in a patient who also had additional hypomethylation at the *MEST/PEG1* DMR and birth growth restriction with persistent short stature in a patient hypomethylated at the *IGF2R* and *ZNF331* DMRs (19).

## Chromosome 15q11: PWS/AS

PWS is characterized by severe hypotonia and feeding difficulties in early infancy, followed in later infancy or early childhood by excessive eating and gradual development of morbid obesity (unless eating is externally controlled). Motor milestones and language development are delayed. All individuals have some degree of cognitive impairment. A distinctive behavioral phenotype (with temper tantrums, stubbornness, manipulative behavior, and obsessive-compulsive characteristics) is common. Hypogonadism is present in both males and females and manifests as

genital hypoplasia, incomplete pubertal development, and, in most cases, infertility (82–84). The major genetic defect is a *de novo* paternally derived deletion of the chromosomal region 15q11–q13 (70%–75% of patients), followed by maternal UPD (20%–25%) and in rare cases an imprinting defect affecting the paternal chromosome 15 (82). In 10%–15% of patients with an imprinting defect, deletions of the PWS critical region inside the imprinting center (IC) on chromosome 15 have been identified (85, 86).

AS is characterized by severe developmental delay or intellectual disability, severe speech impairment, gait ataxia and/or tremulousness of the limbs, and a unique behavior with an inappropriate happy demeanour that includes frequent laughing, smiling, and excitability. Microcephaly and seizures are also common features. Developmental delays are first noted at around age 6 months; however, the unique clinical features of AS do not become manifest until after age 1 year, and it can take several years before the correct clinical diagnosis is obvious (82, 87, 88). AS is caused by the loss of function of *UBE3A*, which is expressed from the maternal allele in brain. Point mutations in the *UBE3A* gene have been identified (89, 90) in nearly 11% of patients (91, 92). Other disease mechanisms are either a *de novo* maternally derived deletion of 15q11–q13 (70%–75% of cases), paternal UPD (3%–7%), or an imprinting defect affecting the maternal chromosome 15 (~3%) (82, 93). The 10%–15% of the imprinting defects are caused by microdeletions (6–200 kb) that include the AS critical region of the chromosome 15 IC (85).

MLMD is very rare in patients with methylation defects at 15q11, mainly perhaps because very few patients present imprinting defects. In fact, only one case with a molecular diagnosis of AS has been described to have additional imprinting anomalies, affecting ICR2, *PEG3*, and the *GNAS* locus (47, 94). This AS-MLMD patient differed significantly from typical AS, having initially been referred for BWS and PWS testing.

## Concluding remarks

As the increasing number of reports on MLMD shows, it appears to be restricted to TNDM, SRS, BWS, and some cases of PHP1B, whereas this phenomenon has not been described in PWS and just one case with hypomethylation at *SNRPN* (typical of AS). Except for TNDM, where only hypomethylation at maternally imprinted loci has been observed, MLMD can include both hypermethylation and hypomethylation at either paternally or maternally imprinted regions and can differ among the tissues

studied for the same individual. However, it is possible that MLMD is more frequent than known as all studies reported to date are focused on the specific imprinting locus. Future whole-methylome analyses will help us answer this point. Further efforts are needed to identify (i) the putative network involved in the hypermethylation/hypomethylation grouping and (ii) mechanism(s) involved in methylation establishment and maintenance, whose defects are responsible for these IDs. New high-throughput techniques, e.g., (bisulfite) exome/genome sequencing and analyses at chromatin organization level, in combination with functional assays will elucidate the interaction between imprinting control regions/genes and their target(s), contributing to the understanding of mechanism of genomic imprinting and its disturbances.

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