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Multi-locus imprinting disturbances of Beckwith-Wiedemann and Large offspring syndrome/Abnormal offspring syndrome: A brief review

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ABSTRACT

In vitro fertilization and somatic cell nuclear transfer are assisted reproduction technologies commonly used in humans and cattle, respectively. Despite advances in these technologies, molecular failures can occur, increasing the chance of the onset of imprinting disorders in the offspring. Large offspring syndrome/abnormal offspring syndrome (LOS/AOS) has been described in cattle and has features such as hypergrowth, malformation of organs, and skeletal and placental defects. In humans, Beckwith-Wiedemann syndrome (BWS) has phenotypic characteristics similar to those found in LOS/AOS. In both syndromes, disruption of genomic imprinting associated with loss of parental-specific expression and parental-specific epigenetic marks is involved in the molecular etiology. Changes in the imprinting pattern of these genes lead to loss of imprinting (LOI) due to gain or loss of methylation, inducing the emergence of these syndromes. Several studies have reported locus-specific alterations in these syndromes, such as hypomethylation in imprinting control region 2 (KvDMR1) in BWS and LOS/AOS. These LOI events can occur at multiple imprinted loci in the same affected individual, which are called multi-locus methylation defect (MLMD) events. Although the bovine species has been proposed as a developmental model for human imprinting disorders, there is little information on bovine imprinted genes in the literature, even the correlation of epimutation data with clinical characteristics. In this study, we performed a systematic review of all the multi-locus LOI events described in human BWS and LOS/AOS, in order to determine in which imprinted genes the largest changes in the pattern of DNA methylation and expression occur, helping to fill gaps for a better understanding of the etiology of both syndromes.

1. Introduction

Assisted reproductive biotechnologies such as *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI) and somatic cell nuclear transfer (SCNT) have been shown to have many scientific and biotechnological applications in recent decades [1]. In the Western world, approximately 4–6% of the population uses IVF and ICSI as a means of human reproduction [2]. However, less than 50% of the IVF embryos generally reach the blastocyst stage. In turn, SCNT in cattle has much lower efficiency, with an approximate success rate of 10%–15%, indicating a higher failure rate during embryonic development [3].

Protocols of SCNT and IVF have been widely used in many species of domestic animals [4]. Among all domestic species, the bovine is considered the best animal model for the nuclear transfer technique, since it presents the highest nuclear transfer efficiency rate. In addition, the bovine SCNT offspring can present epigenetic alterations similar to those observed in children conceived by IVF, making cattle a good developmental animal model for human applications [4,5].

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Embryos generated by assisted reproduction technologies (ARTs) present abnormalities at different stages of embryonic development as well as in the postnatal period, which can indicate epigenetic failures, such as changes in the normal DNA methylation and histone modification patterns at imprinted loci [6].

Abnormal expression of genes regulated by genomic imprinting may affect embryo development and compromise the success of implantation [7]. One of the most prevalent epigenetic markers for establishing the genomic imprinting pattern is DNA methylation. Alterations in DNA methylation status are associated with the etiology of some syndromes arising from ARTs leading to Refs. [8,9]. Some studies have reported changes in multiple imprinted genes due to alterations in the DNA methylation pattern [10–12]. This scenario leads to multi-locus imprinting disturbance (MLID) caused by multi-locus methylation defects (MLMD).

In humans and cattle, multi-locus imprinting disorders due to ARTs may increase the rate of developing congenital overgrowth syndromes. For example, the Beckwith-Wiedemann syndrome (BWS), in humans is associated with an up to nine-fold increase in the risk of the child being born with the syndrome [13]. In bovines, the fetuses produced *in vitro* show morphological abnormalities such as changes in the placenta and fetus, associated with hypergrowth. These changes lead to increased embryonic loss and perinatal mortality leading to the occurrence of Large offspring syndrome/abnormal offspring syndrome (LOS/AOS) [1].

The molecular interspecific similarities with bovine species make them an ideal model for comparison with human imprinting defects arising after submitting gametes and embryos to ARTs. BWS and LOS/AOS are associated with well-established changes in DNA methylation in imprinting control region 2 (ICR2), located in the gene cluster of the antisense transcript *KCNQ1OT1* (KvDMR1), presenting loss of DNA methylation (LOM) [14]. This LOM causes deregulation of 12 DMRs of imprinted genes located in this cluster. Another altered genomic imprinting region is the ICR1, which controls the *H19-IGF2* cluster. In BWS patients, the gain in DNA methylation (GOM) in ICR1 leads to an increase of *IGF2* expression, which plays an important role as a growth factor [15].

Although there are similarities in MLMD in some imprinted genes such as *KCNQ1OT1*, *H19* and *PLAGL1*, there are still gaps in analysis of multi-locus failures in BWS and LOS/AOS, since some studies have not investigated the same loci (Fig. 1). Besides that, there is no study in LOS/AOS demonstrating the correlation of the multi-locus alteration pattern in imprinted genes with the phenotypic data observed in animals.

In humans, imprinted genes are better documented than in bovines, leading to further multi-locus studies. In addition, there are studies of BWS that associate the alteration of MLMD in imprinted genes with the phenotypic data of the patients [14,16].

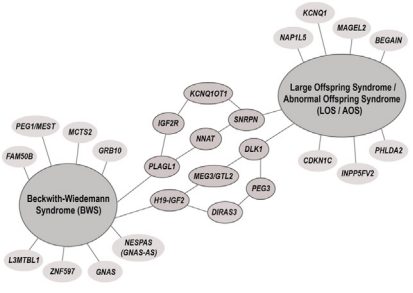

Fig. 1. Imprinted genes involved in BWS and LOS/AOS. Multi-locus characteristics of BWS and LOS/AOS (light gray), in addition to the altered genes in common (dark gray), demonstrating the mosaicism of DNA methylation changes in the imprinted genes present in both, as in *KCNQ1OT1*, *PLAGL1*, *H19* and *SNRPN*.

This has not yet been reported in LOS/AOS. In turn, studies in cattle allow analyzes of different tissues, which is a limitation in humans.

Thus, the objective of this review was to carry out an assessment of reports in the literature of multi-locus alteration in imprinted genes in both syndromes to identify the gaps preventing their better understanding. In cattle, these studies' focus has been on animals produced through SCNT, since the occurrence of LOS/AOS in these animals has higher frequency than in those produced via IVF [1].

After the analysis, only those studies that evaluated at least three genes regulated by genomic imprinting were considered as involving multi-locus methylation defects (MLMD). We found 19 articles describing at least the occurrence of MLMDs in both species, with the co-occurrence of epigenetic changes in 11 loci. The major findings report GOM and LOM events in almost all loci from individuals conceived through ART protocols.

2. Genomic imprinting

In mammals, a set of genes is expressed from only one of the parental alleles through the mechanism of genomic imprinting, due to the differential silencing during gametic development [17]. Genetic information obtained from the Geneimprint database (http://www.geneimprint.com/) demonstrates that more than a 100 human genes are regulated by genomic imprinting [18,19], while in cattle approximately 30 genes are well described as regulated by this mechanism [5,20]. This difference demonstrates a relative dearth of studies of imprinted genes in cattle, especially regarding their location and expression pattern.

These genes are evenly distributed on all chromosomes of both species, but the exact functions of some imprinted genes not well described, although it is known they play a fundamental role in embryonic, placental and postnatal development, besides having neurological and behavioral functions. Interestingly, it is postulated that imprinted genes expressed by the maternal allele play a role in repressing fetal growth, so that the genes expressed paternally act to increase growth [21].

The imprinted genes are usually located in clusters along the genome through regulation by DNA elements. These clusters are called the imprinting control regions (ICRs), which are capable of regulating three to twelve genes [21,22]. These regions anchor parental-specific patterns of DNA methylation and histone modifications, causing these ICRs to become differentially methylated regions (DMRs).

The establishment of DMRs during development also affects their role in the imprinting pattern, in which the deletion of a gametic DMR causes the loss of imprinting (LOI) of several genes within the cluster [23]. The loss of a somatic DMR can only affect the expression of an imprinted gene within the cluster, so the imprinting pattern of the other cluster genes is maintained [21]. Underlying the differential epigenetic marks at the DMRs, a large variety of regulatory elements such as enhancers, insulators and others participate in the regulation of transcription of imprinted genes, to induce specific parental expression in other genes, mainly genes located in the same cluster [21].

During early embryo development, the genome undergoes nuclear reprogramming, in which the gametic epigenome marks are erased and *de novo* epigenetic programming occurs, establishing the epigenome of the embryo [18]. However, imprinted genes escape this wave of reprogramming through the action of cis and trans acting factors, supporting the hypothesis that these correct regulations of genes are fundamental in mammals' embryonic development [22].

The establishment and maintenance of allelic specific DNA methylation in ICRs is a complex mechanism that involves many

regulatory factors, which can be affected by environmental factors such as exposure to *in vitro* cell culture or physiological changes [1,24]. Studies of various species have reported changes in different imprinted loci simultaneously in genetic syndromes, associated with gamete and/or embryo exposure to reproductive technologies [7,25,26]. These changes at different chromosomal regions are called multi-locus imprinting disorders (MIDs), which arise due to multi-locus changes in DNA methylation in the imprinted genes [8,9].

3. Influence of reproductive biotechnologies on the genomic imprinting mechanism

It is already known that assisted reproductive technology protocols induce epigenetic changes in imprinted genes [27]. In different biotechnological approaches, the cell manipulation steps, nuclear donor cell sources, nuclear activation protocol, and different culture media can have negative effects during embryo development, contributing to LOI occurrence and causing different imprinting disorders in the offspring [2,28]. Many studies corroborate that manipulation of the *in vitro* environment causes changes in mRNA expression and genomic imprinting patterns [5,12,29]. Failure of the imprinting pattern in bovine clones is evidence of errors in epigenetic reprogramming after fertilization [6].

In animal models, different studies have shown that the effect of *in vitro* culture on embryonic development causes molecular instability in imprinted gene regulation, loss of monoallelic transcription, and changes in DNA methylation [2]. Some studies have also shown that assisted reproduction technologies (ARTs) can increase the risk of syndromes associated with changes in intrauterine growth, defects in placental formation, prolonged gestation, postnatal death, and breathing difficulties [24,28].

Imprinting disorders (IDs) may arise due to different changes in the genome and epigenome, such as: duplications/deletions, uniparental disomy (UPD), epimutations, and point mutations in imprinted genes [30]. IDs are associated with clinical characteristics such as postnatal growth retardation, postnatal hypo and hypergrowth, neonatal hyperglycemia, and behavioral changes [31,32]. Recently, IDs involving changes in imprinting patterns in multiple loci, characterized as multi-locus methylation defects (MLMDs), have emerged as topics in a new group of studies, called MIDs [9,33].

Due to MIDs, patients with a certain epimutation such as hypomethylation of ICR2 in BWS may present changes in other loci regulated by imprinting [16,30,31]. More than 50% of patients with MIDs have epimutations in DNA methylation due to alterations in cis and trans regulating factors [16]. Most multi-locus imprinting disorders detected have similar clinical and molecular characteristics due to epimutations in the same loci of imprinted genes [34]. The initial studies that detected MLMD in ID syndromes identified changes in at least two imprinting loci, in ID transient neonatal diabetes mellitus (TNDM) and Beckwith-Wiedemann syndrome [35,36].

Today, the best known IDs in humans are the Beckwith-Wiedemann, Silver-Russell (SRS), Prader-Willi, Angelman, and TNDM syndromes, while the most common disorder in the bovine species is LOS/AOS [5,31,37]. Recently, many studies have proven the association between these syndromes, especially BWS, SRS and TNDM, and MLMD events [8,38].

Since many imprinted genes have not been investigated in more detail, it is possible that MLMDs affect more patients than reported in the literature, also considering that the expression pattern of these genes may be tissue-specific [34].

Many genes have already been associated with these multi-locus failure events, such as *ATA3*, *IMPACT*, *L3MBTL*, *MAGEL2*, *MKRN3/ZNF127*, *PEG3*, *SNRPNR*, *UBE3A*, *ZAC1/PLAGL1*, *PEG1/MEST*, *GRB10*, and *GNAS*, in addition to the better-described *H19/IGF2* and *KCNQ1OT1* [12,31,34]. In BWS and LOS/AOS many imprinted genes analyzed are involved in the success of placental, fetal and postnatal development, despite there is still a lack of information about the imprinting pattern of certain genes (Table 1).

Most changes in the imprinting pattern in these genes are due to hypomethylation, with the exception of some patients with SRS, who present hypermethylation of DNA [8]. Although these IDs have this molecular heterogeneity, the functional relevance of the loss (LOM) or gain (GOM) of methylation in affected patients is still unknown, specifically what determines the frequency of different phenotypes in these syndromes [39]. Thus, the appearance of multi-locus changes in imprinted genes in human and bovine species is indicative of how complex the mechanism of establishment and maintenance of the imprinting pattern is, so further studies are needed to elucidate how these occur.

3.1. Beckwith-Wiedemann syndrome

Beckwith-Wiedemann is a hypergrowth syndrome and the clinical features may vary due to the predominant molecular mosaicism [14]. The phenotypic characteristics are associated with hemihyperplasia, increased risk of tumors, and abdominal wall defects [15,40]. Other clinical features are organomegaly, macrosomia, macroglossia, neonatal hypoglycemia, renal abnormalities, and craniofacial dimorphism [17,30,31,33].

The detection and diagnosis of the syndrome is important due to the risk of formation of tumors such as Wilms' tumor, hepatoblastoma, and even neuroblastoma. The risk of a child with BWS presenting some type of tumor is up to 7.5% in the first eight years of life, and up to 21% over the lifetime [14]. The verification of tumors is important in these patients regardless of the molecular etiology. However, the clinical diagnosis of the syndrome may be difficult due to molecular mosaicism owing to the presence of cases with MLMDs.

Thus, the etiology of BWS is complex due to altered expression and the DNA methylation pattern of imprinted genes located on the human chromosome 11p15. The main cause of BWS occurrence is (50%–60%) owing to epimutations in the chromosome 11p15 cluster [14]. The chromosome 11p15 cluster comprises two ICRs that regulate domains of imprinted genes: ICR1 and ICR2. ICR1 contains the *H19* and *IGF2* genes, which are transcribed by the maternal and paternal alleles, respectively [14]. The *IGF2* gene encodes a protein that has a growth factor function in tumors. In contrast, the *H19* gene acts as a tumor suppressor [41]. The ICR1 controls the specific parental monoallelic expression of *H19* and *IGF2*, through the joint action of insulators and enhancers. BWS patients can present GOM on ICR1, resulting in biallelic expression of the *IGF2* gene, where about 2%–7% of patients are affected [15,41].

ICR2 covers 10 genes regulated by genomic imprinting, some of which present alteration on BWS syndrome, such as *KCNQ1OT1*, *CDKN1C* and *KCNQ1* genes [9]. The *KCNQ1OT1* (LIT1) gene encodes an lncRNA paternally expressed associated with epigenetic regulation of other imprinted genes in the ICR2 domain [41]. In BWS patients, the hypomethylation of ICR2 induces the expression of the maternal allele of this gene. LOM in ICR2 occurs in approximately 50%–60% in BWS patients [31,37]. The *KCNQ1* gene is expressed maternally, and the change in its expression pattern increases the incidence of tumors in BWS patients. Finally, the *CDKN1C* gene, also expressed maternally, has the function of regulating cell proliferation altered in up to 5% of BWS cases [31].

Besides the epigenetic alterations in both ICRs, other molecular changes can occur in BWS patients. Paternal UPD involving ICRs 1

Table 1
Imprinted genes involved in the etiology of BWS and LOS/AOS. Location of imprinted genes in humans and cattle and their function. The imprinted genes are well-distributed on all chromosomes and the pattern of imprinting is similar in both species.

Genes	Human	Bovine	Function
	Location/Expressed allele	Location/ Expressed allele	
KCNQ1OT1	chromosome 11/ Paternal	chromosome 29/ Paternal	The *KCNQ1OT1*, an lncRNA that regulates a bidirectional silencing at KCNQ1 cluster genes.
KCNQ1	chromosome 11/ Maternal	chromosome 29/ Maternal	The *KCNQ1* gene provides directions for potassium channels, acting on the cell's ability to generate and transmit electrical signals.
H19	chromosome 11/ Maternal	chromosome 29/ Maternal	The *H19* gene produces a noncoding RNA. *H19* gene acts as a tumor suppressor and has an important role in early development.
IGF2	chromosome 11/ Paternal	chromosome 29/ Paternal	The *IGF2* gene encodes a protein that plays an essential role in fetal growth and development.
IGF2R	chromosome 6/ Maternal	chromosome 9/ Maternal	The *IGF2R* encodes the IGF2 receptor. This receptor acting in the intracellular trafficking of lysosomal enzymes, the TGF-β activation, and *IGF2* degradation.
PEG1/MEST	chromosome 7/ Paternal	chromosome 4/ Paternal	The *PEG1/MEST* gene is an adipocyte enlargement factor. Also, the LOI of this gene is related to embryonic lethality.
PLAGL1	chromosome 6/ Paternal	chromosome 9/ Paternal	The *PLAGL1* gene encodes a zinc finger protein associated with tumor suppression and cell apoptosis regulation.
SNRPN	chromosome 15/ Paternal	chromosome 21/ Paternal	The *SNRPN* gene encodes a component of the small nuclear ribonucleoprotein complex, which plays a role in pre-mRNA processing and may contribute to tissue-specific alternative splicing.
MEG3/GTL2	chromosome 14/ Maternal	chromosome 21/ Maternal	The *MEG3* gene encodes a lncRNA and is expressed in many normal tissues but its expression is lost in multiple cancer cell lines of various tissue origins. It is suggested that this gene is a lncRNA tumor suppressor.
NAP1L5	chromosome 4/ Paternal	chromosome 6/ Paternal	The *NAP1L5* gene encodes a protein that shares sequence similarity to nucleosome assembly factors. *NAP1L2* is a paralog of this gene, in which is expressed in neural tissues.
DIRAS3	chromosome 1/ Paternal	chromosome 3/ Paternal	The *DIRAS3* gene encodes a member of the *ras* superfamily and acts as a tumor suppressor. The re-expression of this gene in cancer cells is associated with inhibits growth, decreases invasiveness, and induces apoptosis.
NNAT	chromosome 20/ Paternal	chromosome 13/ Paternal	The *NNAT* gene encodes two proteolipids translated from differentially spliced mRNAs. This gene act as a regulation of ion channels, in Ca-channels particularly. Besides, this gene presents a role in forming and maintaining the structure of the nervous system.
DLK1	chromosome 14/ Paternal	chromosome 21/ Paternal	The *DLK1* encodes a transmembrane protein that makes as a regulator of cell growth.
GRB10	chromosome 7/Isoform Dependent	chromosome 4/[a]	The *GRB10* gene encodes three splice variants with different functionalities. The Grb10 product interacts with a number of receptor tyrosine kinases and signaling molecules, besides participate in growth control, insulin signaling and cellular proliferation. As *GRB10* gene has isoform dependent expression, its function may change depending on the allele being expressed.
FAM50B	chromosome 6/ Paternal	chromosome X/[a]	The *FAM50B* gene encodes an antisense transcript, FAM50B-AS, which present an role in spermatogenesis and tumorigenesis.
PEG10	chromosome 7/ Paternal	chromosome 4/ Paternal	The *PEG10* is derived from a Ty3/gypsy long terminal repeat (LTR) retro-transposon, but it does not work as an active retrotransposon. It is expressed in embryonic tissues, mainly in the placenta, and has a role to enhance cell proliferation and blocks apoptosis.
ZNF597	chromosome 16/ Maternal	chromosome 25/ Maternal	The *ZNF597* gene encodes a zinc finger protein. This biological function is unknown.
L3MBTL1	chromosome 20/ Paternal	[a]/[a]	The L*3MBTL1* represents a polycomb group gene. The encoded protein functions to regulate gene activity, likely via chromatin modification. *L3MBTL1* acts by regulating p53-dependent quality control systems that degrade misfolded proteins. Reports showed that this gene probably plays a role in cell proliferation.
INPP5F V2	chromosome 10/ Paternal	chromosome 26/ Paternal	The *INPP5F* gene encodes an inositol phosphatase protein. The transcript *INPP5F V2* is paternally expressed only in the brains. This gene plays a role in endocytic recycling and is also involved in cell migration mechanisms.
GNAS	chromosome 20/ Isoform Dependent	chromosome 13/ Maternal	The *GNAS* gene generates multiples gene products that triggers a complex network of signaling pathways that influence many cell functions by regulating the activity of hormones. Mutations on the GNAS gene is related to endocrine and non-endocrine tumors. Also, the change in the DNA methylation pattern of the maternal allele in this gene is related to pseudohypoparathyroidism (PHP)-Ia.
NESPAS (GNAS-AS1)	chromosome 20/ Paternal	[a]/[a]	The *NESPAS* gene, also known as *GNAS-AS1*, encodes an antisense RNA transcript that plays a role in the GNAS complex locus regulation.
PEG3	chromosome 19/ Paternal	chromosome 26/ Paternal	The *PEG3* gene encodes different transcript variants with distinct isoforms. This gene plays a role in cell proliferation and p53-mediated apoptosis. Also, has a tumor suppressing activity in glioma cells.
MAGEL2	chromosome 15/ Paternal	chromosome 21/ Paternal	The *MAGEL2* gene encodes the melanoma-antigen-subfamily-like-2 protein. This gene is part of a ubiquitination complex that acts in regulates endocytosis, receptor recycling, and cell-surface localization.
BEGAIN	chromosome 14/[a]	chromosome 21/ Paternal	The *BEGAIN* gene encodes a neuronal protein that plays a role related pathways are Protein-protein interactions at synapses. This gene forms a complex with PSD-95 may sustain the structure of postsynaptic density (PSD).

[a] Unreported or conflicting data in the literature and/or database.

and 2 is predominant in up to 20% of patients [15,24]. Rare cases (approximately 1%) are a result of duplication of regions on chromosome 11p15 [13,14,42]. In ART cases, the predominant epimutation in BWS children is also LOM from ICR2 [17].

Various studies in recent decades observed that about 25% patients with LOM in the *KCNQ1OT1* gene also have changes in other imprinted loci, such as in the *PLAGL1* and *GNAS* genes [15,40,43,44]. Moreover, the occurrence of LOI events was also demonstrated at multiple imprinting loci in BWS patients conceived by both natural and assisted reproduction [45]. The occurrence of MLMD in BWS is higher in patients conceived through ART, with *GNAS*, *PLAGL1*, *PEG1/MEST*, *IGF2R*, and *GRB10* being the most affected genes [26,46,47].

The MLMD event in BWS cases can occur because of changes in cis and trans factors. A recent study showed that a mutation in a gene that encodes a transcription factor, *ZFP57*, which serves to maintain the imprinting pattern in the post-fertilization period, was involved in the MLMD event [48]. Mutations in other genes such as *NLPR2* and *NLPR7*, have already been associated with cases of BWS with epimutations in more than two imprinted loci [49].

The incidence of certain clinical features in BWS can vary from patient to patient. The greater occurrence of Wilms' tumors in patients is due to epimutations in ICR1, affecting approximately 5% of patients with changes in ICR2 [14]. This occurs because the altered molecular mechanisms may be different in each case. Some changes, such as UPD, occur only in the post-zygotic phase, which induces greater phenotypic variability in some patients who have multi-locus methylation changes in the imprinted genes [41].

Corroborating with the aforementioned data, the clinical characteristics of MLMD patients are the outcome of multi-locus methylation defects affecting different tissues [23]. Although there are studies on MLMD in BWS, there is little data on the frequency that this multi-locus event occurs and what are the mechanisms involved.

3.2. Large offspring syndrome (LOS)/Abnormal offspring syndrome (AOS) as a model for Beckwith-Wiedemann syndrome

Similar to BWS, LOS/AOS is an overgrowth syndrome, with clinical features such as organomegaly, breathing difficulties, immunological deficiencies, increased fetal and neonatal death rate, and malformation of the upper and lower limbs [12,50].

The greatest embryo loss in cattle from ARTs occurs mainly in the implantation period and in the first trimester of pregnancy [51]. This is due to fetal loss during early embryonic development, between the 30th and 90th day of implantation [51]. Developmental failures can occur at the end of embryogenesis, enabling birth of the progeny, but survival occurs only for a few hours due to poor organ formation [1]. Thus, fetal loss during pregnancy has high rates in bovine cloning [51].

In recent years, bovine embryos produced *in vitro* have been used as a study model for human syndromes related to LOI events [52]. This is attributable to similarities phenotypic aspects and pre-implantation development, such as gene activation, cell metabolism, and interaction with the culture medium [29]. Studies in MLMD events have focused in multiple imprinted genes associated with BWS and LOS/AOS, but there are still gaps to be filled regarding the molecular etiology of both syndromes (Fig. 1).

The clinical features present in BWS may vary depending on the predominant epimutation. This type of correlation has not yet been performed for LOS/AOS. There are some properties that allow the use of bovine syndrome as a model for human BWS, including the clinical similarities between the syndromes, the increased expression of IGF2 observed in clones with LOS/AOS [29], and the high conservation between the imprinted genes regulation in the two species, which is not observed between mice and humans [52]. Other similarities between the two species are in the conservation of the pattern of expression and alteration of DNA methylation in the two syndromes, as well as other parameters during embryonic development, such as gestation time and progeny size [52], although there is still a need in LOS/AOS to correlate molecular data with the predominant phenotypic characteristics in animals.

4. Multi-locus profile of DNA methylation in BWS and LOS/AOS

The MLMD event can occur in imprinted loci carrying both maternal or paternal methylation profiles [31]. This indicates that the LOI may arises in the post-fertilization period, causing abnormalities in maintaining the imprinting profile of the genes. The degree of demethylation in the affected loci does not always explain the dominant characteristic phenotype of these syndromes in multi-locus patients [24].

The mosaic of MLMD event indicates that epigenetic errors emerge after fertilization, in the second wave of reprogramming. The mechanisms involved in epigenetic failures at different loci in multi-locus patients are distinct from those restricted to one locus only [45].

One the pioneers studies the multi-locus events in imprinting syndromes investigated the methylation pattern in four imprinted genes (*IGF2R*, *PEG1/MEST*, *H19* and *SNPRN*) in 40 patients with BWS that already had LOM in the *KCNQ1OT1* region [45] (Table 2). Among the patients, 11 were conceived by ART and 29 naturally. After analysis, it was observed that three of the 11 patients with BWS from ART presented abnormal methylation in at least one of the analyzed loci. Only the *PEG1/MEST* gene showed a normal methylation pattern in BWS ART patients. Of the naturally conceived BWS patients, only seven (~24%) had abnormal methylation in at least one imprinting locus. Although some patients had a multi-locus event, there was no phenotypic difference between patients with epigenetic changes in a single locus and multiple loci.

In 2009, multi-locus changes in human BWS and SRS syndromes were analyzed in seven genes regulated by genomic imprinting (*IGF2R*, *PEG1/MEST*, *KCNQ1OT1*, *PLAGL1/ZAC*, *SNRPN*, *DLK1-GTL2*, *H19* and *IGF2*) [24]. This study was the first to report the presence of multi-locus changes in BWS patients who already had LOM in ICR2 [24]. In BWS, among patients with LOM in ICR2, 24% also had LOM in another locus and 62.5% in two other imprinting loci, as in the *PLAGL1/ZAC*, *PEG1/MEST* and *SNRPN* genes. The H19-DMR showed GOM in patients with BWS. Only the *DLK1/GTL2* region did not have epimutations in patients with BWS. Although the presence of multi-locus changes was described, there were no differences in phenotypic characteristics in the different groups of patients with or without multi-locus characteristics. Despite the occurrence of multi-locus characteristics in the majority of the patients analyzed, the authors found no significant difference between patients conceived by ART with one locus and multiple loci [24].

A cohort study of 149 patients with BWS, in which 81 had hypomethylation in ICR2, analyzed the DNA methylation pattern in 11 other DMRs. This analysis showed that hypomethylation affected several imprinting loci, not being restricted to ICR2, and that all altered DMRs had maternal methylation. Partial and incomplete hypomethylation was found, being an indicative of the presence of imprinting mosaicism after zygote formation [40].

In a study where 25 BWS patients conceived by ART protocols (IVF and ICSI) were analyzed, 24 showed LOM in ICR2 (96%) [43]. In this same study, 87 naturally conceived BWS patients analyzed, 100% showed LOM in ICR2 [43]. The authors observed other imprinting loci, and in the ART group at least three patients had LOM in the *PEG1/MEST* and *SNRPN* loci. Similarly, in the natural reproduction group, LOM was also noted in the *PEG1/MEST* and *PLAGL1/ZAC* loci. The frequency of LOM in ART patients (37.5%) was significantly higher than in patients from natural reproduction (6.4%) [43].

Analysis in a BWS patient specifically with LOM in KvDMR1 did not show any alteration in any other analyzed genes (*IGF2R*, *PLAGL1/ZAC*, *GRB10*, *PEG1/MEST*, *H19*, and *MEG3*) [53]. In the same year, 15 BWS patients were analyzed of which almost 50% had LOM in DMRs associated with the *KCNQ1OT1* gene. Of the total number of patients evaluated, only one was from ART (ICSI), where multi-locus methylation changes were observed in the *PEG1/MEST* (GOM) and *NESPAS* (LOM) genes [10].

A study evaluating only BWS patients generated by natural reproduction showed that 33% (14/42) had hypomethylation in ICR2. Of this total, three patients also had hypermethylation in the *GNAS/NESP* cluster, and the others had hypomethylation in other analyzed genes (*DIRAS3*, *GRB10*, *PEG1/MEST*, *PLAGL1/ZAC*) [47].

A study of 28 patients found that 12 were diagnosed with phenotypic characteristics of BWS, and presented hypomethylation of *PLAGL1* and *IGF2R*, but these were not characterized as MLMDs [54].

Table 2
MLMD pattern in assisted reproduction BWS patients. Survey of 14 studies with BWS patients from ART. Characterization of MLMD pattern represented with LOM and GOM highlighted with light and dark gray tones, respectively. Imprinted genes with marked black squares represents the unaltered DNA methylation pattern according to some studies. The remaining squares demonstrate that there was no analysis of DNA methylation in these imprinted genes in the 14 BWS studies.

Genes	Imprinted genes DNA methylation pattern (BWS)														References
KCNQ1OT1	LOM	LOM	LOM	LOM	LOM	LOM	LOM	LOM	LOM	LOM	LOM	LOM	LOM	LOM	[10,11,24–26,40,43,45–47,53–56]
H19		GOM	GOM		■										[24,40,53]
IGF2	LOM	GOM													[24,45]
IGF2R		LOM	LOM		■		LOM		LOM	LOM	LOM	LOM			[24–26,40,46,47,53,54]
PEG1/MEST	■	LOM	LOM	LOM	■	GOM	LOM	LOM	LOM	LOM	LOM		LOM	LOM	[10,11,24–26,40,43,45–47,53,55,56]
PLAGL1		LOM	LOM	LOM	■		LOM	LOM	LOM	LOM	LOM	LOM	LOM		[24–26,40,43,46,47,53–56]
SNRPN	LOM	LOM	■	LOM									LOM	LOM	[11,24,40,43,45,56]
GNAS			LOM				GOM	LOM		LOM	LOM			LOM	[11,25,40,46,47,55]
NESPAS			LOM			LOM				LOM	LOM				[10,25,40,46]
GRB10			■		■									LOM	[11,40,53]
DIRAS3							LOM			■	LOM		LOM	LOM	[11,25,46,47,56]
GLT2/MEG3		■	■		■										[24,40,53]
L3MTBL							LOM			■	LOM				[25,46,47]
FAMB50B										■	LOM		LOM		[25,46,56]
PEG3			■												[40]
MCTS2											GOM				[25]
NNAT											LOM				[25]
DLK1		■													[24]
PEG13							LOM								[47]
ZNF597							GOM								[47]

Abbreviations: LOM: Loss of Methylation / GOM: Gain of Methylation / MLMD: Multi-locus Methylation Defects
Abbreviations: LOM: Loss of Methylation/GOM: Gain of Methylation/MLMD: Multi-locus Methylation Defects.

Many studies have demonstrated that patients with BWS generated by assisted reproduction present a picture of multi-locus methylation alteration in at least three imprinting loci when it already contains KvDMR1 hypomethylation, with a greater tendency to LOM in maternal DMRs [11,25,26,37,46,55,56].

In bovines, the first study of alteration of multi-locus methylation was published in 2011, in which the authors evaluated the level of methylation in bovine liver tissue generated by SCNT. After evaluating the DMR of three imprinted genes located in the same cluster (GTL2-DMR, IG-DMR and DLK1-DMR), it was observed the predominant pattern of hypermethylation in the two clones that died shortly after birth and in two control animals. The control sample used in the study came from IVF, which would explain the pattern of hypermethylation similar to that found in the SCNT samples [57] (Table 3).

In contrast to BWS studies, DNA methylation of the *H19*, *IGF2*, and *XIST* genes of six bovine clones had a pattern of methylation loss in ICR1 in different tissues (kidney, heart, lung, and others). These findings occurred after treatment with 5-aza-2 (¢) -deoxycytidine (5-Aza-dc) [58] (Table 3).

To date, Rocio Melissa Rivera's research group at the University of Missouri (USA) has presented the most complete studies on MLMD events in bovine clones. In the studies, a hypomethylation pattern was observed in several imprinting loci (*KCNQ1OT1*, *H19/IGF2*, *CDKN1C*, *PLAGL1*, and *SNRPN*) similar to that described by other works. The *PHLDA2*, *BEGAIN*, *DIRAS3*, *MAGEL2*, *NAP1L5*, *PEG3*, *NNAT*, and *INPP5F* genes also had LOM in their DMRs. This pattern of LOI was observed in kidney, muscle, brain, liver, placenta and tongue tissues [12,29].

5. Multi-locus expression pattern in BWS and LOS/AOS

The specific parental monoallelic expression of genes regulated by imprinting plays a crucial role during embryonic development, and therefore can be used as genetic markers of embryo viability [25]. Most genes regulated by imprinting have a function in several biological processes, such as metabolism, acting as transcription factors, cell cycle regulators in apoptosis, among other processes [3]. A few studies have analyzed this parameter in LOS/AOS animals, whereas in BWS this type of analysis is still lacking.

Thus, in cattle some studies demonstrate alteration of multi-locus expression, such as the analysis of three imprinted genes (*IGF2*, *H19*, and *IGF2R*) in eight tissues (kidney, liver, heart, lung, bladder, spleen, thymus and muscle) from five post-death bovine clones, and in three tissues (skin, muscle and liver) of six adult clones and five control animals from natural reproduction. Analysis from quantitative RT-PCR showed that in clones that did not survive, the expression level was altered in all the evaluated genes compared with the expression observed in the control animals [4].

The expression of these genes in adult clones showed normality in all tissues, except the *IGF2* gene in the muscle, where one of the clones presented 10-fold greater expression of the *IGF2* gene in heart, liver, and spleen tissues compared to controls, indicating the *IGF2* was overexpressed. For the *H19* gene, the same occurred, in which one of the clones demonstrated roughly a 30-fold increase in the expression of this gene in liver tissue. Although all clones presented altered expression, the study did not find a relationship between the level of expression and the phenotypic characteristic of the analyzed clones [4].

Another study demonstrated alteration of multi-locus expression in bovine clones in liver, brain, tongue, heart and chorioallantoic tissues, in different imprinted genes (*PLAGL1*, *H19*, *KCNQ1OT1*, *CDKN1C*). However, in their analysis on D65 clones, no expression change was observed, with all genes showing monoallelic expression (Table 4) [52].

In contrast, analyses performed on tissues of different

Table 3
Tissue-specific multi-locus of DNA methylation pattern in bovine conceived from SCNT. Characterization the pattern of LOM (light gray) and GOM (dark gray) in the different imprinted genes, according to previous studies. The remaining squares demonstrate that there was no analysis of DNA methylation in these imprinted genes in these studies.

Genes	Kidney	Liver	Placenta	Muscle	Brain	Tongue	References
DLK1		GOM					[57]
IG-DMR		GOM					
GTL2		GOM					
KCNQ1OT1	LOM	LOM	LOM			LOM	[29]
CDKN1C				LOM	LOM		
KCNQ1OT1	LOM						[12]
MAGEL2	LOM						
BEGAIN	LOM						
DIRAS3	LOM						
NAP1L5	LOM						
NNAT	LOM	LOM		LOM	LOM		
PEG3	LOM						
IGF2R	LOM	LOM		LOM			
PLAGL1	LOM			LOM	LOM		
SNRPN	LOM				LOM		

Abbreviations: LOM: Loss of Methylation / GOM: Gain of Methylation / SCNT: Somatic Cell Nuclear Transfer
Abbreviations: LOM: Loss of Methylation/GOM: Gain of Methylation/SCNT: Somatic Cell Nuclear Transfer.

embryonic origins (liver, kidney, brain, placenta, muscle, tongue) demonstrated a monoallelic and biallelic expression pattern in several imprinting loci in animals with LOS/AOS, which is associated with LOM and GOM. Kidney, muscle and liver tissues showed biallelic expression of the *KCNQ1OT1*, *PHLDA2*, *KCNQ1*, *BEGAIN*, *DIRAS3*, *IGF2R*, and *NNAT* genes [12,29].

Recently, a transcriptome was generated for four bovine clones with LOS and four control fetuses using the RNA-seq technique in different tissues (skeletal muscle, liver, kidney, brain) in order to identify differentially expressed genes (DEGs) [59]. The number of DEGs varied between tissues, in which the clones that presented LOM in *KCNQ1OT1* had a higher rate of DEGs in the liver and skeletal muscle. Clones 1, 3, and 4 showed higher DEGs in liver, kidney and skeletal muscle tissues, respectively. This tissue-specific expression mosaicism demonstrates the need for further characterization of the expression pattern of different genes in tissues.

This same study also evaluated the relationship between DEGs and biological function in different tissues. The authors observed that the biological functions varied between tissues, in which functions such as “cancer metabolic pathways” and “oxidative

Table 4
Multi-locus expression pattern in SCNT clones. Monoallelic (light gray) and biallelic (dark gray) expression in specific tissues in different genomic imprinted genes, characteristic of LOS/AOS. (*) monoallelic expression; (*/*) biallelic expression. The remaining squares demonstrate that there was no analysis of imprinted genes expression in these studies.

Genes/Tissues	Kidney	Liver	Placenta	Muscle	Brain	Tongue	Heart	Lung	Chorioallantoic	Reference
KCNQ1OT1		*			*	*	*		*	[52]
CDKN1C		*			*	*	*		*	
PLAGL1		*			*	*	*		*	
H19		*			*	*	*		*	
KCNQ1OT1	*/*	*/*	*	*/*	*/*	*/*	*/*	*/*		[29]
KCNQ1	*/*	*/*	*/*	*/*	*/*	*/*	*/*	*/*		
CDKN1C	*	*	*	*	*/*	*	*	*		
PHLDA2	*/*	*	*	*/*	*/*	*/*	*/*	*/*		
H19	*	*	*	*	*	*	*	*		
IGF2	*	*	*	*	*	*	*	*		
KCNQ1OT1	*/*									[12]
BEGAIN	*/*									
DIRAS3	*/*									
MAGEL2	*/*									
NAP1L5	*/*									
PEG3	*/*									
IGF2R	*/*	*/*		*/*						
SNRPN	*/*									
INPP5FV2					*/*					
NNAT	*/*			*/*						
PLAGL1	*/*			*	*					

Abbreviations: SCNT: Somatic Cell Nuclear Transfer; LOS/AOS: Large Offspring Syndrome/Abnormal Offspring Syndrome
Abbreviations: SCNT: Somatic Cell Nuclear Transfer; LOS/AOS: Large Offspring Syndrome/Abnormal Offspring Syndrome.

phosphorylation" were predominant in the skeletal muscle and liver, respectively. From this association, it was possible to assess which genes had altered expression in the clones' tissues, such as the *IGF2* gene, which was associated with the metabolic pathways of cancer [59].

In that study, the researchers also performed whole genome bisulfite sequencing to identify skeletal muscle imprinting regions in the clones. DMRs were found in 18 imprinted genes, seven of which had already been described in cattle (GNAS-DMR, H19/IGF2-DMR, GNASXL-DMR, IGF2R/AIRN-DMR, KvDMR1, PEG1/MEST-DMR, and PLAGL1-DMR), eight new genes already described in humans and mice have been identified (NAP1L5-DMR, DIRAS3-DMR, GTL2-DMR and DLK1-DMR), and three new regions have been detected (BEGAIN-DMR, MEG-DMR 8, and MGC157368-DMR). The DMRs of the newly detected imprinted genes were located within gene bodies of known bovine imprinted genes [59].

6. Conclusions and future perspectives

According to the findings described, the similarity in the phenotypic and epigenetic pattern between LOS/AOS and BWS is evident, which justifies the use of the bovine species for studies on the etiology of BWS. However, more detailed studies are needed for multi-locus characterization of DNA methylation changes, in addition to other factors that might contribute to the etiology of these syndromes. Further studies in cattle should analyze other imprinting clusters since there is a huge gap between the description of imprinted genes in humans and cattle. Moreover, there are still no report in LOS/AOS regarding the correlation of epimutation data with clinical characteristics. Multi-locus specific tissue data has not yet been reported in BWS, given the impossibility of collecting this type of material. Thus, our study demonstrates gaps to be explored in studies of LOS/AOS as an animal model for BWS and for a better understanding of their etiology.

Authorship contribution statement

Paula Magnelli Mangiavacchi: Conceptualization, Investigation, Meta-analysis, Methodology, Writing - original draft. **Maria Clara Caldas Bussiere:** Project administration, Supervision, Writing - Review and Editing. **Mariana da S. Mendonça:** Investigation, Methodology, Writing - original draft. **Angelo José Burla Dias:** Writing - Review and Editing. **Alvaro F. Lopes Rios:** Project administration, Supervision, Conceptualization, Methodology, Writing - Review and Editing.

Declaration of competing interest

The authors declare no potential conflicts of interest.

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