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CLINICAL AND MOLECULAR CHARACTERIZATION OF BECKWITH-WIEDEMANN SYNDROME

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Preface

This thesis is a collection of several projects mainly related to Beckwith-Wiedemann syndrome (BWS, OMIM #130650) I had the opportunity to develop during my Ph.D. program. BWS is the most common congenital overgrowth disorder (1 in 10.500 live births) [Mussa et al. 2013], and the paradigm of overgrowth conditions with cancer predisposition. Besides BWS is the model of disorders caused by disruption of genomic imprinting, a process consisting in a parent-of-origin specific gene expression. The projects aim to exploring different molecular and clinical aspects of this condition. The research areas of this dissertation range from a deepening of the complex molecular bases of this wide variable disorder to an expanding of the clinical phenotype, investigating fetal growth pattern, cancer risk, adulthood clinical topics and correlation with assisted reproductive technology.

SECTION I

INTRODUCTION

BECKWITH-WIEDEMANN SYNDROME

Beckwith-Wiedemann syndrome (BWS, OMIM # 130650) is the most common congenital overgrowth disorder (1 in 10500 live births) [Mussa et al. 2013] and is the paradigm of disorders caused by disruption of genomic imprinting. It is also the paradigm of overgrowth conditions with cancer predisposition. It was first clinically described by Beckwith and by Wiedemann in the 1960s [Beckwith 1963, Wiedemann 1964], while the first molecular definition was in the 1990s, with the discovering of epigenetic anomalies in chromosome 11p15.5 region [Hatada et al. 1996, Henry et al. 1991, Reik et al. 1995]. BWS has wide clinical spectrum including several variably associated anomalies: its cardinal features, beside overgrowth, include abdominal wall defects, macroglossia, nephrourologic malformations, hemihyperplasia, hyperinsulinemic hypoglycemic, ear anomalies, hemangiomas and nevus flammeus at the glabella, and organomegaly [Shuman et al. 1993-2013]. Another cardinal feature of BWS is cancer risk, that has been estimated in international literature being between 5 and 15% [Rump et al 2005]. Clinical features can be variably associated and can present with different degrees of severity, characterizing therefore the widely variable clinical spectrum of the disorder. A recent international consensus statement refers to this condition as Beckwith-Wiedemann spectrum (BWSp) and BWS-related phenotypes [Brioude et al. 2018]. This wide clinical range includes also isolated lateralised overgrowth (ILO) [Kalish et al 2017] (previously called ‘isolated hemihypertophy’ or ‘isolated hemihyperplasia’; OMIM #235000) and patients with a chromosome 11p15.5 molecular anomaly who do not fit into these first two groups, a condition termed ‘atypical BWS’ [Brioude et al. 2018].

The diagnosis can be established clinically although none clinical feature is mandatory [Weksberg et al. 2010]. The variability of BWS clinical spectrum is paralleled by comparable (epi)genetic heterogeneity at the molecular level [Choufani et al 2013, Eggerman et al 2014].

Clinical features

BWS clinical features include:

Macroglossia: the most common feature in BWS, found in 90-97% of patients representing the more sensitive trait for BWS identification [Elliott et al., 1994; Gaston et al., 2001; Ibrahim et al., 2014]. Relating to macroglossia severity, patients could have feeding, respiratory and/or language problems. According a recent study macroglossia could be the only clinical feature in 25% of cases and its presence is associated to a high detection rate of molecular analysis [Prada et al. 2012, Elliott et al 1994].

Macrosomia and post-natal overgrowth: recently definded as height and/or weight >2 SDS [Brioude et al. 2018] has been considered the cardinal feature of BWS. However, macrosomia at birth is present only in approximately half of the cases [Brioude et al. 2018]. It appears an almost constant finding in IC1- GoM cases, while post-natal overgrowth is more typical of IC2-LoM cases. Newborns typically present relative microcephaly. Mean adult height in BWS is significantly increased respect to the normal population.

Abdominal wall defects: they can range in severity from omphalocele to umbilical hernia and diastasis recti. Severe abdominal wall defects are associated with centromeric molecular defects [Blohm et al., 1998; Ibrahim et al., 2014].

Lateralized overgrowth: it consists in the abnormal cell proliferation leading to a marked increase in the length and/or girth of most or all of one side of the body compared to its contralateral side. When it involves the legs or trunk, it may result in leg length discrepancy and may be responsible for scoliosis. Leg discrepancy is usually not evident at birth but may develop during the first years of life. Lateralized ovregrowth is the feature showing the strongest association with tumor development, representing a robust clinical predictor of malignancy [Cooper et al., 2005; DeBaun et al., 1998; Ibrahim et al., 2014). It is more frequently found in association with UPD [Brioude et al., 2013; Cooper et al., 2005; Ibrahim et al., 2014].

Organomegaly: organ enlargement can involve liver, kidneys, spleen, thymus, heart, pancreas, and adrenal glands. Fetal adrenocortical cytomegaly is a pathognomonic histologic finding. Organ enlargement represent a risk factor for cancer development [DeBaun et al., 1998; Mussa et al., 2012] and is more frequently observed in IC1-GoM patients [Mussa et al., 2012].

Nephrourological anomalies are present in 28-61% of BWS patients [DeBaun et al., 1998; Goldman et al., 2002; Mussa et al. 2012] and include a range of findings: nephromegaly, cortical/ pyramidal hyperechogenicity and kidney malformations with or without hydronephrosis (double collecting system, megaloureter, caliceal diverticula) are the most common features in newborns and in early infancy while cortical and medullary cysts, nephrocalcinosis and nephrolithiasis (with or without hypercalciuria) usually develop in infancy or adolescence [Goldman et al. 2002]. Ureteral enlargement with vescicoureteral reflux may be associated with polyhydramnios during pregnancy. Severe forms can account for infections in newborns and early childhood, which represent one of the most frequent complication during childhood [Mussa et al., 2012]. A variety of less common urologic findings have been documented in patients with BWS: cryptorchidism is a non-specific feature frequently observed in BWS males and associated with major abdominal wall defects [Mussa et al. 2012].

Hypoglycaemia: is defined as plasma glucose levels <50 mg/dL for the first 6 hours of life and <60 mg/dL thereafter. It occurs in approximately half of BWS newborns and, in the majority of them, it resolves spontaneously. In a small subset of cases it can be persistent needing aggressive treatment (diazoxide, pancreatectomy). When severe and not promptly recognized in newborns, it can be responsible for brain injury and negative neurodevelopmental outcome. Transient hypoglycaemia as a suggestive feature is defined by the above criteria lasting less than a week.

Hyperinsulinism is defined as a glucose infusion rate of ≥8 mg/kg/min, a detectable level of insulin and/or C-peptide and undetectable levels of ketones and free fatty acids. Hyperinsulinism is classified as a cardinal feature when lasting beyond one week and requiring escalated treatment, and as a suggestive feature when lasting less than a week.

Facial appearance includes midface hypoplasia, infraorbital creases, naevus flammeus at the glabella, macroglossia and prominent mandible, anterior earlobe creases and posterior helical pits relative microcephaly with metopic ridge. (Figure 1)

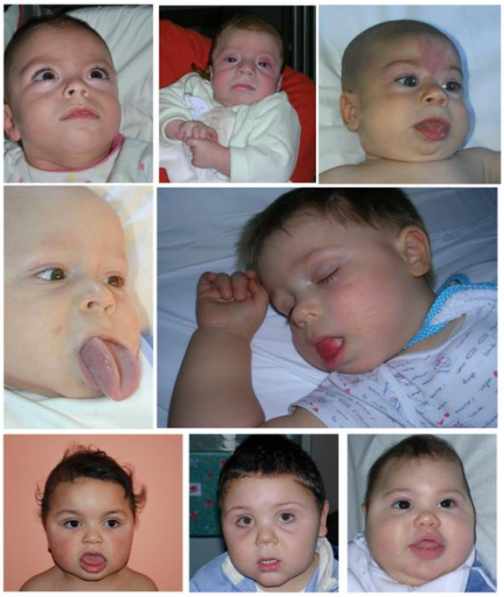


Figure 1. Facial features of BWS patients

Cardiac anomalies are described in 20% of children with BWS, mostly consisting in mild cardiomegaly [Pettenati et al. 1986] or mild to moderate anatomic anomalies such as patent ductus arteriosus or foramen ovale; these defects usually resolve spontaneously. Cardiac rhythm abnormalities (congenital long QT syndrome) have been anecdotally reported in genomic events involving the IC2 [Gurrieri et al. 2013] and malformations were recently found in 4 out of 57 CDKN1C mutated ones [Brioude et al. 2015].

Miscellaneous and additional features include polydactyly, genital abnormalities, extra nipples, and cleft palate. These findings are more frequently observed in CDKN1C mutated cases [Brioude et al. 2015; Gurrieri et al., 2013; Romanelli et al., 2010]; therefore in presence of such features it is recommended to begin the molecular analysis from CDKN1C gene sequencing analysis to optimize the laboratory workup.

Neurological and developmental implications: neuropsychomotor development of BWS children is usually normal [Elliott et al., 1994]. Cases caused by complex chromosomal rearrangements may present additional features besides those typical of BWS, with unpredictable effects on neurologic development. Paternally derived chromosome 11p15.5 duplications are typically associated with intellectual disability [Slavotinek et al., 1997]. Serious perinatal complications connected with prematurity

and severe malformations, particularly misrecognized severe hypoglycemia, further account for secondary impairment of the developmental outcome and may be associated with any molecular defect. Recently, posterior fossa malformations have been described in small subgroups of patients in association with IC2-LoM [Gardiner et al., 2012] or CDKN1C mutation [Brioude et al., 2015].

Embryonal tumor predisposition: the cumulative risk of embryonal tumor during the first decade of life is estimated in 8-10% [Rump et al., 2005]. Neoplasms typically develop during infancy with a likelihood maximum at birth progressively declining during the first 10 years of age to the risk observed in the general population. Wilms' tumor represents the most common histotype (43%), followed by hepatoblastoma (20%), adrenal adenoma/carcinoma (7%), neuroblastoma, rhabdomyosarcoma, pancreatoblastoma, leukemia. BWS molecular subgroups are characterized by relevant differences in cancer risk and occurrence of histotype [Brioude et al., 2013; Cooper et al., 2005; Ibrahim et al., 2014; Rump et al., 2005]. Because of the oncogenic risk patients are submitted to a specific tumor surveillance protocol aiming at the early diagnosis of these tumors.

Although BWS might present prenatally or in adult life, it is most commonly diagnosed in the neonatal period or in early childhood.

BWS diagnosis is clinical and based on criteria that have changed over past years: each BWS feature has a different predictive value, sensitivity and specificity [Ibrahim et al., 2014]. New clinical score system have been recently approved by an International Consensus Statement [Brioude et al. 2018]: a score of ≥4 must be reached for a diagnosis of classical BWS. Children who meet these criteria would be considered to have BWSp, even if an 11p15 anomaly is not identified. (Table 1). The goal of this BWSp scoring system was to recognise that BWS falls into a clinical spectrum and that some features that have long considered to be classical parts of the syndrome are not present in every patient, and therefore the diagnosis should not be dismissed due to the absence of such features. Additionally, this consensus statement sought to include elements that could be pathognomonic for BWS.

Cardinal features are considered key to the clinical diagnosis, whereas suggestive features add to the likelihood of a clinical diagnosis and the indications for molecular testing, but are less specific.

Table 1. Clinical score system [Brioude et al. 2018].

Clinical features of Beckwith–Wiedemann Spectrum

Cardinal features (2 points per feature)	Suggestive features (1 point per feature)
Macroglossia	Birth weight >2 SDS above the mean
Exomphalos	Facial naevus simplex
Lateralised overgrowth	Polyhydramnios and/or Placentomegaly
Multifocal and/or bilateral Wilms tumour or nephroblastomatosis	Ear creases and/or pits
Hyperinsulinism (lasting beyond one week and requiring escalated treatment)	Transient hypoglycaemia (lasting less than a week)
Pathology findings:adrenal cortex cytomegaly, placental mesenchymal dysplasia orpancreatic adenomatosis	Typical BWSp tumours (neuroblastoma, rhabdomyosarcoma, unilateral Wilms tumour, hepatoblastoma, adrenocortical carcinoma or phaeochromocytoma)
-	Nephromegaly and/or Hepatomegaly
-	Umbilical hernia and/or diastasis recti

For clinical diagnosis of classical BWS a patient requires a score of >/= 4. Patients with a score of >/=2 require genetic testing. Patients with a score >2 do not meet the criteria for genetic testing.

Molecular tests may confirm the diagnosis, help the geneticist to provide appropriate counseling, and the clinician to assess cancer risk. Negative molecular tests cannot rule out BWS, as low-grade mosaicism for molecular defects may not be detectable with the diagnostic tests commonly employed. It should also taken into account the possibility that so far unrecognized genetic causes of BWS are present in a minority of patients.

So it has been introduced the concept of Beckwith-Wiedemann Spectrum (BWSp) (Figure 2) that includes patients with a clinical diagnosis of Beckwith–Wiedemann syndrome (BWS) with or without an (epi)genetic change at the BWS locus on chromosome 11p15; patients with ‘atypical BWS’ (defined as fewer cardinal and suggestive features than those needed for a clinical diagnosis of BWS) and an (epi)genetic change at the BWS locus; and patients with ‘isolated lateralised overgrowth’ and an (epi)genetic change at the BWS locus.

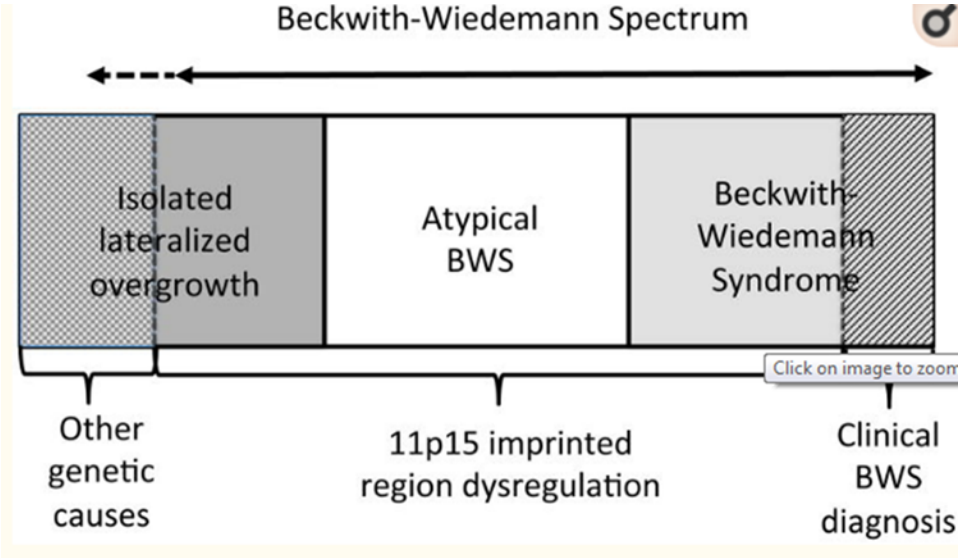


Figure 2. Clinical spectrum of 11p15 region dysregulation [Brioude F. et al. 2018]

Molecular features

The molecular etiology of BWS is complex involving alterations of the expression of multiple imprinted growth regulatory genes on chromosome 11p15.5 [Weksberg et al., 2005]. Most autosomal genes are normally expressed from both the paternal and maternal allele. However, genes that are imprinted are expressed predominantly or exclusively from either the maternal or paternal allele in a parent of origin- specific manner. That is, some imprinted genes are expressed from the paternal allele while the maternal copy is silenced and, conversely, others are expressed from the maternal allele while the paternal allele is silenced. Genomic imprinting is regulated by epigenetic mechanisms (extrinsic to changes in primary nucleotide sequence) that include DNA methylation, histone modification, and noncoding RNAs. ICs are regions of DNA that regulate the expression of imprinted genes in *cis* over large distances and show differential methylation of the parental alleles. Therefore, they are also termed differentially methylated regions (DMRs).

Epigenomic and genomic alterations in the imprinting cluster on chromosome 11p15 are detected in up to 80% of BWS patients using currently available testing methodologies [Weksberg et al., 2010]. This chromosomal region can be divided into two distinct regulatory domains (IC1 and IC2) (Figure 3) separated by a non-imprinted region and contains two clusters of genes involved in cellular cycle and somatic growth control. IC1 and IC2 are characterized by differential methylation of their maternal and paternal

alleles: in normal conditions, IC1 of the paternal allele and IC2 of the maternal allele are methylated.

The telomeric/distal domain 1 (IC1) contains the imprinted genes insulin-like growth factor 2 (IGF2) and H19. IC1 is methylated on the paternal allele during spermatogenesis and is unmethylated on the maternal allele. The maternally expressed H19 gene encodes a 2.3-kb untranslated RNAPolII transcript. H19 is processed into small microRNAs [Cai and Cullen, 2007] one of which, miR-675, negatively regulates the expression of IGF-1 receptor [Keniry et al., 2012]. Loss of H19 expression is not lethal in mice, and such animals display a phenotype of organ overgrowth similar to infants with BWS [Leighton et al., 1995]. H19 has been proposed to function as a tumor suppressor [Hao et al., 1993]. Furthermore, overexpression of H19 is associated with pre- and postnatal growth restriction phenotype [Guo et al., 2008] as well as Russell–Silver syndrome (RSS) [Eggermann, 2010]. The IGF2 gene encodes a paternally expressed cytokine that plays an important role as a growth factor. IC1 regulates imprinted expression of H19 and IGF2: on the maternal chromosome, IC1 is unmethylated, permitting the transcription of H19 while on the paternal chromosome, methylation of IC1 prevents binding of the CTCF protein to IC1, so that the IGF2 promoter has access to the downstream enhancers and is expressed while H19 is silenced [Hark et al., 2000].

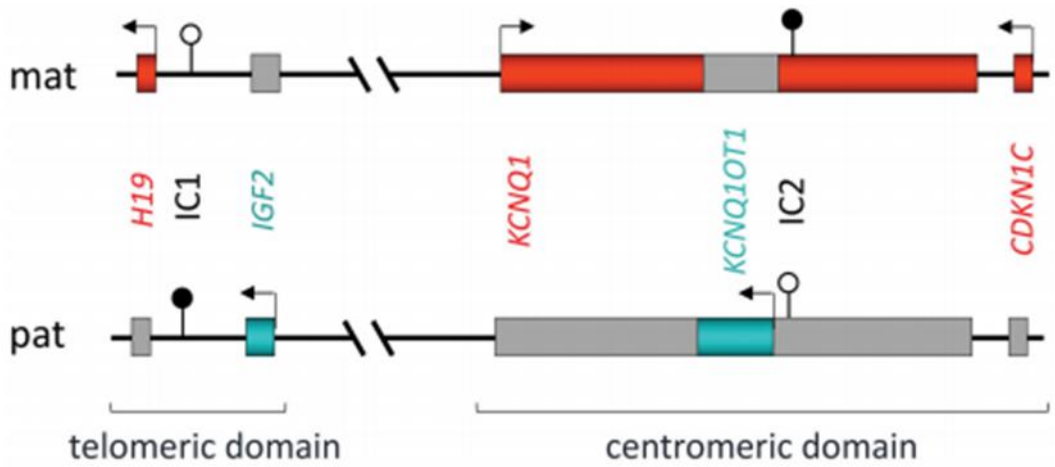
Molecular alterations limited to domain 1 occur in 5-10% of patients with BWS. These include gain of methylation at IC1, that is, methylation occurs at both the maternal and the paternal IC1. This change in methylation results in biallelic expression of the IGF2 gene (normally expressed by the paternal allele) and reduced expression of the oncosuppressor H19 gene (normally expressed by the maternal allele). [WN et al 2005].

In the centromeric/proximal domain 2 (IC2), the human imprinted cluster spans a 1Mb region containing approximately 10 imprinted genes. Two of these genes, KCNQ1OT1 and CDKN1C, are clearly implicated in BWS [Kunze and Wiedemann, 1993]. CDKN1C gene encodes a cyclin-dependent kinase inhibitor that regulates prenatal and postnatal growth and development and is normally expressed by the maternal allele [Matsuoka S. et al., 1995]. Sporadic loss of methylation at IC2 occurs in 50% of patients [Weksber R e Shuman C. 2000] leading to reduced expression of CDKN1C.

Mutations in CDKN1C accounts for5% of patients with BWS but this frequency increases to 40% of familial cases [Li et al 2001].

Paternal uniparental disomy (UPD) involving chromosome 11p15 is found in 20% of cases and usually encompasses both imprinted gene clusters (IC1 and IC2). The extent of the UPD varies in different patients but complete paternal UPD for chromosome 11 is extremely rare. Interestingly, the vast majority of BWS cases with UPD (paternal) demonstrate somatic mosaicism. Therefore, UPD occurs in BWS is post-zygotic somatic recombination. These data also suggest that normal imprinted gene expression in this chromosomal region is a requirement for normal early embryonic development [Choufani S et al. 2013].

Figure 3. Complex BWS molecular mechanisms [Brioude F. et 2018].



Rarely (1%) cytogenetically detectable anomalies give rise to the BWS phenotype [Weksberg et al. 2005]. Duplications involve the paternally derived chromosome 11, whereas translocations and inversions associated with the BWS phenotype involve the maternally derived chromosome 11 [Cardarelli et al., 2010]. Estimates for recurrence risk for parents of a child with a duplication involving 11p15.5 would depend on their karyotype status. The recurrence risk for BWS is 50% for cases of maternal transmission of a pathogenic CDKN1C mutation while in cases due to abnormal methylation of IC1 and IC2 domains or paternal UPD recurrence risk I similar to general population.

Approximately 10-15% of clinically diagnosed individuals do not have a detectable molecular defect, even if they have an evident phenotype: these patients could present an unknown molecular mechanism or a tissue mosaicism level undetectable by current technologies. Table 2 summarizes BWSp molecular defect categories and recurrence risk [Brioude F. et al. 2018]

Table 2. BWSp molecular defect categories

Molecular defect	Frequency of molecular defect	Mosaicism observed	Risk of recurrence	Characteristic clinical features (compared with other molecular subgroups)
IC1 GOM	5%[48]	Yes[26,54,76,78,81]	• If no genetic anomaly is present, <1%[28] • If genetic anomaly (for example, pathogenic SNV of copy number variant in the DMR) is present, 50%; dependent on parental origin[71,73,105,106]	• Low frequency of exomphalos[10,13,16] • High risk of Wilms tumour[13,58,149]
IC2 LOM	50%[48]	Yes[26,54,76,78,81]	• If no genetic anomaly is identified, <1%[28] • If a *cis*-acting genetic anomaly is present, 50%; dependent on parental origin[99–103]	• High frequency of exomphalos[10,13,16] • Low risk of Wilms tumour[13,58,149]
upd(11)pat	20%[48] (see also paternal uniploidy)	Yes[26,54,61,76,78,81]	<1%[28]	• High incidence of lateralised overgrowth[10,13] • Low frequency of exomphalos[10,13,16] • High risk of Wilms tumour and hepatoblastoma[13,58,149]
Loss-of-function *CDKN1C* variants	5% (40% in familial cases)[48]	Usually no, but has been reported rarely[83]	50% on maternal transmission[82,83]	• High frequency of exomphalos[10,13,16] • Low risk of Wilms tumour[13,58,149]
Dup(11)(p15.5)pat	~2–4%[55]	No[55]	• 50% on paternal transmission[55,94] • Risk for SRS on maternal transmission[57,95,96]	
Deletions involving 11p15	1–5%[55,98]	No[55]	Dependent on extent and position of CNV, and parent of origin[55]	
Mosaic paternal unidiploidy (Genomewide paternal UPD)	Up to 10% of upd(11)pat[62–67,184]	Yes[62–67,184]	Low[62–67,184]	High frequency of neoplasia[63,64,137,152]
MLID	33% of IC2 LOM cases[75–78,88]	Yes[78,88–91]	Low, unless an *in trans* genetic variant is identified[79,80]	Unclear[75,76,78,89,92,93]

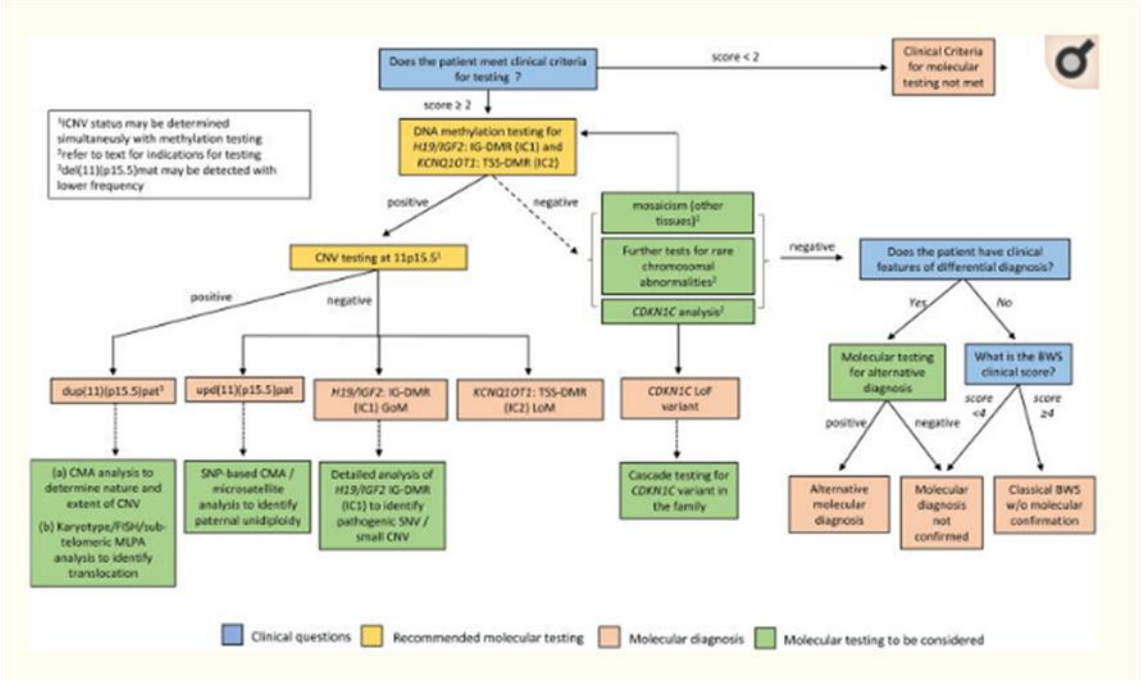
Molecular analysis

Methylation-sensitive multiplex ligation probe analysis (MS-MLPA) is currently the most robust method for detecting the majority of epigenetic and genetic etiologies associated with BWS. It detects microdeletions/microduplications, alterations in gene

dosage, and DNA methylation including UPD [Scott RH et al.2008]. UPD can be suspected by methylation sensitive techniques and needs to be confirmed by microsatellite analysis or SNP array as the somatic mosaicism associated with this etiology may lead to weak signals on MS-MLPA. Moreover, failure to detect UPD11 in one tissue (usually leukocytes) is not conclusive. One should consider obtaining another tissue (such as skin), especially in the event of surgery. Karyotype analysis will detect the rare *de novo* and maternally transmitted translocations/inversions (1%) and paternally derived duplications (1%) of chromosome 11p15.5 associated with BWS. Translocation/inversions almost always disrupt the gene, KCNQ1, and are not usually detectable by MLPA because most do not show DNA copy number changes or DNA methylation changes. Finally, DNA sequencing is required to detect genomic alterations in CDKN1C associated with BWS. The CDKN1C ($p57^{kip2}$) mutations are seen both sporadically (5%) and in autosomal dominant pedigrees modified by preferential parent of origin-specific transmission (40%) [Weksberg R. et al. 2010]. So these tests are usually performed in tandem, beginning from methylation analysis, given its higher likelihood of leading to a genetic confirmation of the diagnostic suspect. In familial cases, the diagnostic process should begin from the CDKN1C gene sequencing and standard karyotyping.

The consensus group recommended that molecular testing is indicated in cases with a score of ≥2, unless there is an alternative explanation (for example, gestational diabetes mellitus for macrosomia). For isolated exomphalos, molecular testing is discretionary. Testing is recommended in patients with a family history and a known heritable pathogenic 11p15 anomaly (a positive family history might occur in 10–15% of patients) [Brioude et al. 2018]. Figure 4 summarizes the molecular diagnostic pathway for investigation of suspectd BWSp.

Figure 4. International Consensus Statement diagnostic flow chart [Brioude et al. 2018]



To date, our cohort consists of 108 patients with a clinical diagnosis of BWS and 49 patients with diagnosis of Isolated Lateralized Overgrowth (ILO). Detailed clinical information were routinely collected from clinical records and anamnestic investigation. Table 3 and figure 5 and 6summarize the genotypic spectrum of our cohort.

Table 3. Genotypic spectrum of our BWS and ILO cohorts.

MOLECULAR MECHANISM	**N° AFFECTED INDIVIDUALS**		**%**	
	BWS	**ILO**	**BWS**	**ILO**
LoM-IC2	27/108	1/49	25%	2%
GoM-IC1	6/108	-	5.5%	
UPDpat	16/108	1/49	14.8%	2%
CDKN1C mutations	3/108	-	2.7%	

Molecular analysis refused	3/108	-	2.7%	
Molecular analysis in progress	1/108	6/49	0.9%	12.2%

Figure 5. Genotypic spectrum of our BWS cohort

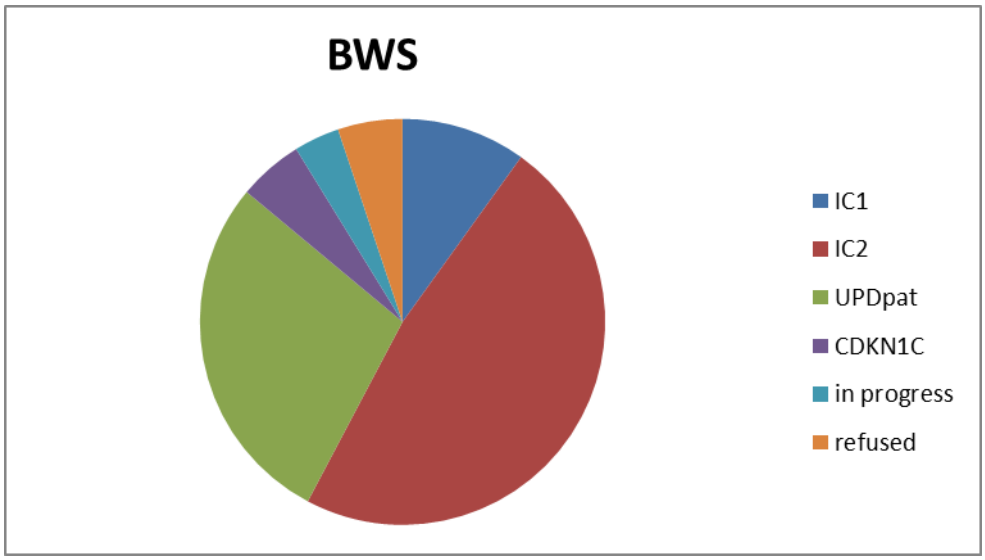
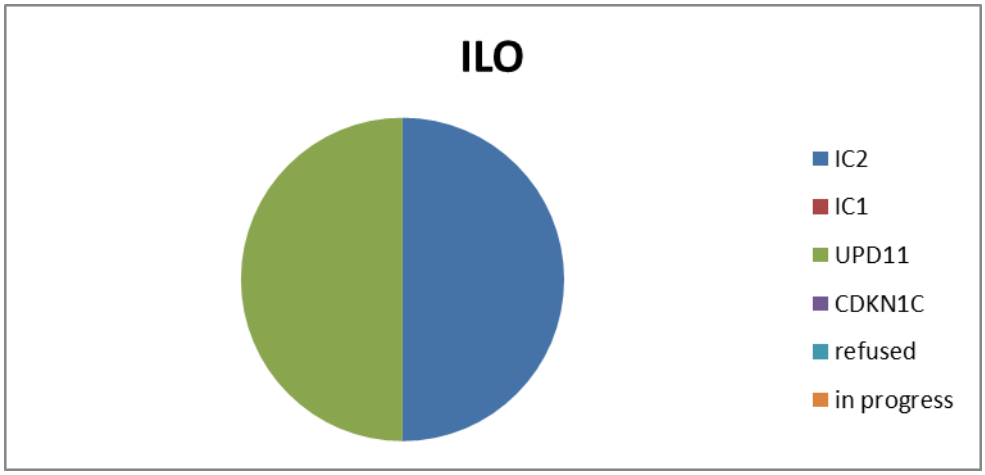


Figure 6. Genotypic spectrum of our ILO cohort



SECTION II

INVESTIGATIONAL CONTRIBUTIONS

Chapter 1.

Deepening the molecular basis and their consequences in Beckwith-Wiedemann syndrome: definition of epigenotype-phenotype correlations

This work has been published as

*Mussa A, Russo S, De Crescenzo A, Freschi A, Calzari L, Maitz S, Macchiaiolo M, Molinatto C, Baldassarre G, Mariani M, Tarani L, Bedeschi MF, Milani D, Melis D, Bartuli A, Cubellis MV, Selicorni A, Cirillo Silengo M, Larizza L, Riccio A, Ferrero GB.***(Epi)genotype-phenotype correlations in Beckwith-Wiedemann syndrome.** Eur J Hum Genet. 2016 Feb;24(2):183-90.

BACKGROUND

In preface we outlight how the wide clinical spectrum of BWS is paralleled by comparable (epi)genetic heterogeneity at the molecule level.

Although different investigations proved the association between molecular alterations, clinical features, and cancer risk [Rump et al. 2005, Cooper et al. 2005, Brioude et al. 2013, Choufani et al. 2010, Ibrahim et al. 2014], the complex (epi)geotype-phenotype relationship in BWS has still to be fully unraveled. So we report the clinical and molecular characterization of a large cohort of BWS patients that allows detailed (epi)genotype–phenotype correlations and supports the hypothesis that different (epi)genetic alterations are associated with specific phenotypes in BWS.

Material and methods

Phenotyping

Overall, 318 patients were ascertained via the Italian National BWS Network following referral to the laboratories providing genetic testing for BWS in Italy (Laboratory of Cytogenetics and Molecular Genetics, Istituto Auxologico Italiano, Milan and DiSTABiF, Second University of Naples, Italy). Through the involvement of the major clinical genetics centers, clinical information was collected by the physicians who made the diagnosis, requested the genetic testing, and followed-up of cases. Using a standardized questionnaire, physicians were asked to specify the presence/absence of the features of BWS and provide informations relevant to phenotype and tumor development. Macrosomia was defined as birth weight >90th percentile according to gestational age. Discrete BWS features (eg, macroglossia, hemihyperplasia, nevus flammeus) were diagnosed by evaluation by respective specialists (eg, odontostomatologist, ortopedics, dermatologist). Data were further implemented through a search in the AIEOP (Italian Onco-Hematological Association) tumor registry. Therefore, tumor occurrence is updated to the latest available visit and double checked via a tumor registry allowing a more precise definition of the tumor risk during the follow-up. Patients with at least two BWS criteria (among abdominal wall defects presence and severity, macroglossia, macrosomia, embryonal tumor, ear malformations, organ enlargement, nevus flammeus, hemihyperplasia, nephrourological malformations, cleft palate,

hypoglycemia, family history of BWS, polyhydramnios) and proven molecular diagnosis were included. Four cases with isolated hemihyperplasia and positive molecular tests were also included. To provide a fully meaningful analysis of the correlation between phenotype and (epi)genotype, negative cases were not taken into consideration to avoid ascertainment bias owing to overlapping conditions.

Genotyping

All patients or the parents provided written informed consent to the genetic testing. DNA was extracted from peripheral blood lymphocytes. Methylation analysis of the 11p15.5 chromosomal regions containing IC1 and IC2 was carried out in all patients and performed either by Southern blotting (*n*=170), COBRA (*n*=45) or Methylation-Sensitive Multiple Ligation Probe Amplification (MS-MLPA MRC-HOLLAND kit) (*n*=103). The results obtained by these techniques have been shown to be comparable [Prilo et al. 2008]. In patients with suspected UPD, confirmation was obtained by microsatellite analysis of probands and parents, as described [Russo et al. 2003]. The presence of genome-wide UPD was tested in 28 UPD patients by microsatellite analysis and single-nucleotide polymorphism array. *CDKN1C* gene sequencing as described elsewhere [Romanelli et al. 2010] was carried out in 154 patients selected on the basis of negativity of methylation sensitive tests plus 2 of the above-mentioned BWS diagnostic criteria and either familiarity for BWS or signs/malformations highly specific for *CDKN1C* variants (as palatoschisis or omphalocele). Pathogenicity prediction of *CDKN1C* variants was tested by the bioinformatic tools PolyPhen-2 (Polymorphism Phenotyping), SIFT (Sorting Intolerant From Tolerant), and PROVEAN (Protein Variation Effect Analyzer). Variants were submitted to LOVD (Leiden Open Variation Database 3.0, www.lovd.nl, variants #0000058604, #0000058622, #000005860, #000005862, #0000058601, #0000058602, #0000055971, #0000055979, #0000055977, #0000055899, submitter ID 01227).

Statistical analysis

Data were summarized with descriptive statistics. Comparisons among the molecular groups was conducted by 2 × 2 (for each category. versus all other categories) or comparing categories by 3 × 2 or 4 × 2 Fisher's exact tests or, in case of expected

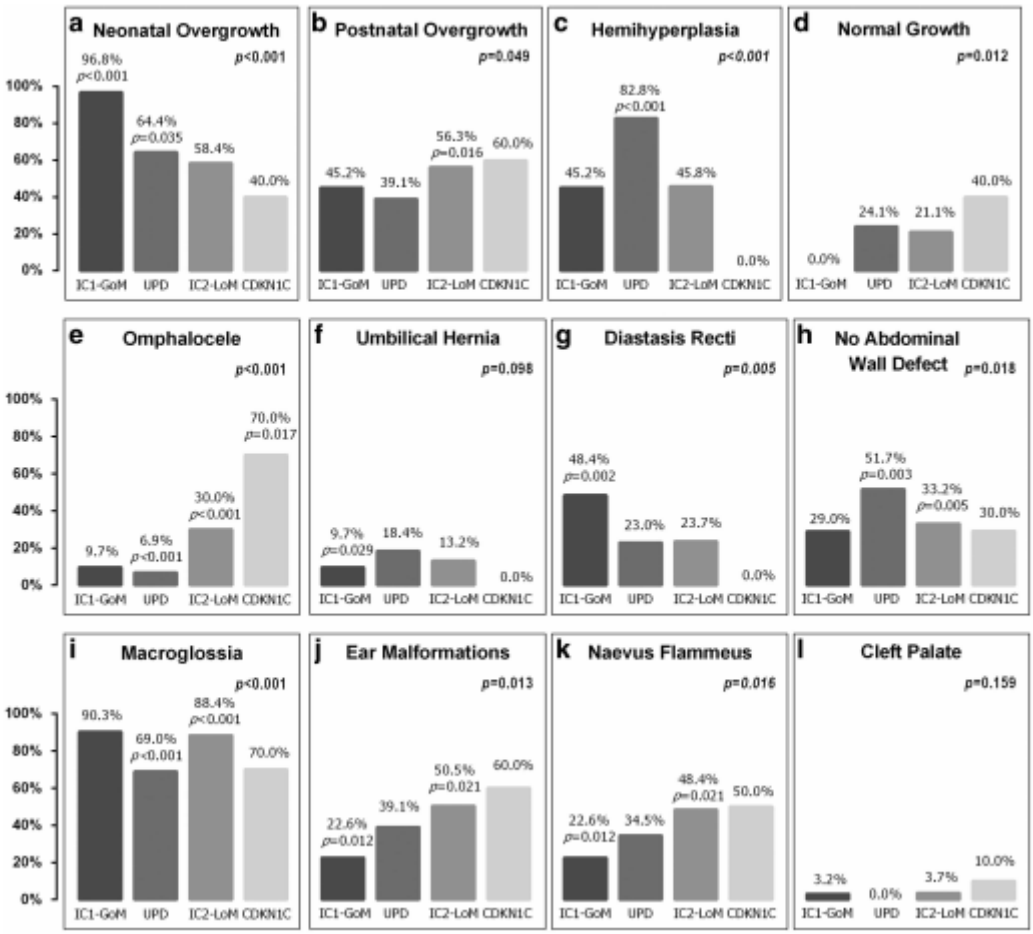
frequencies ≥ 5, χ^2-test with Yates correction, as appropriate. Two-tailed *P*-values <0.05 were considered as significant. Data were analyzed by SPSS 13.0 (IBM Software, Armonk, NY, USA) and Prism GraphPad 5.0 (GraphPad Software, La Jolla, CA, USA).

Results

A total of 318 patients with confirmed epimutation in 11p15.5 or CDKN1C variant were characterized. The following molecular anomalies were identified: 190 IC2-LoM (184 epigenetic anomalies, 5 already published cases with familial IC2 duplications21 and 1 IC2 deletion), 87 UPD carriers, 31 IC1-GoM (21 already published cases22, 23 including one IC1 duplication, one translocation, 11 familial microdeletions24, 25, 26), 10 CDKN1C variants (all unrelated cases, 9 maternally inherited). None of the patients tested was positive for genome-wide UPD. The four cases with isolated hemihyperplasia were affected by UPD (n=2) or IC2-LoM (n=2).

The prevalence of the BWS features in the four subgroups is summarized in Figure 1. The growth patterns showed relevant differences across the molecular subtypes (Figure 1a and d). In patients with IC1-GoM, neonatal macrosomia was almost constant and much more common than in the other subgroups (P=0.002). The prevalence of postnatal overgrowth showed minor differences, being slightly higher in patients with IC2-LoM (P=0.016) and CDKN1C variants and lower in those with UPD (P=0.049). The latter group had an incidence of hemihyperplasia of almost twofold that of IC2-LoM/IC1-GoM patients (P<0.001), whereas hemihyperplasia was not observed at all in CDKN1C variants (P<0.001). Also the distribution of the severity of abdominal wall defects varied extensively among BWS subtypes (Figure 1e and h, Figure 2).Their prevalence was higher in the IC1-GoM group (P<0.001, 70% of cases), in which the defects were mostly minor (P<0.001) with diastasis recti prevailing (P=0.007). Minor defects were also common among UPD patients, but with an overall prevalence of abdominal wall defects much lower than in other groups (48.3%, P<0.001). Patients with IC2-LoM had an intermediate prevalence of abdominal wall defects (66.8%) and showed an increased risk of major ones (omphalocele 30.0%, P<0.001). Patients with CDKN1C variants showed very high incidence of omphalocele (70%, P=0.001). Macroglossia was present in most of the

cases with IC1-GoM (90.3%) and IC2-LoM (88.4%), but was less common in UPD (69.0%) and CDKN1C variant cases (70%) (P<0.001) (Figure 1i). Ear signs were more represented among IC2-LoM and CDKN1C variant patients (50.5% and 60%, respectively) than among IC1-GoM or UPD cases (22.6% and 39.1%, respectively, P=0.013) (Figure 1j). Similar differences were observed for the occurrence of nevus flammeus (48.4%, 50.5%, 22.6%, and 34.5%, respectively, P=0.016) (Figure 1k). Cleft palate was more common in CDKN1C variant patients (Figure 1l), but not significantly. Organ enlargement was reported in 67.7% of IC1-GoM cases, significantly higher than the occurrence in IC2-LoM (27.9%), UPD (36.8%), and CDKN1C variant (10%) cases (P<0.001) (Figure 1m). Kidney abnormalities were more frequently detected in IC1-GoM (32.5%) and UPD (26.4%) patients, as compared with IC2-LoM (8.9%) and CDKN1C (20%) variant cases (P<0.001) (Figure 1n). Ureteral malformations prevalence was highest among IC1-GoM cases (22.6%, P<0.001) and lower in other subtypes (overall 5.2%) (Figure 1o).



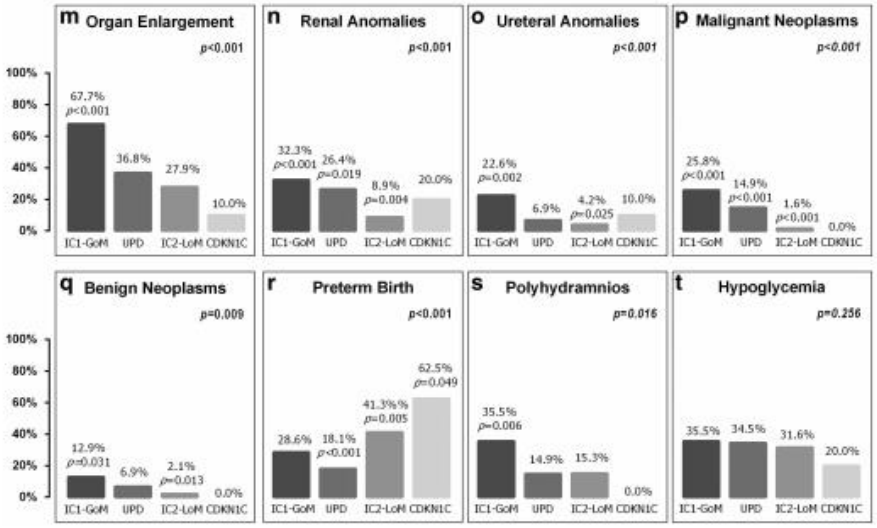


Figure 1 Differences in the prevalence of the features in the molecular subtypes of the syndrome: (**a**) neonatal overgrowth, (**b**) postnatal overgrowth, (**c**) hemihyperplasia, (**d**) normal growth, (**e**) omphalocele, (**f**) umbilical hernia, (**g**) diastasis recti, (**h**) no abdominal wall defect, (**i**) macroglossia, (**j**) ear malformations, (**k**) naevus flammeus, (**l**) cleft palate, (**m**) organ enlargment, (**n**) renal anomalies, (**o**) ureteral anomalies, (**p**) malignant neoplasms, (**q**) benign neoplasms, (**r**) preterm birth, (**s**) polyhydramnios, (**t**) hypoglycemia. *P*-values in the corner of panels refer to the comparison among the four groups, *P*-values above columns to the comparison of each molecular subtype with the other three (non-significant values not shown).

Fourteen (4.4%) patients conceived with the use of assisted reproduction techniques: 10 cases had IC2-LoM and 4 had UPD. Preterm birth (<37 weeks of gestation) was more common in cases with CDKN1C variants (71.4%) and IC2-LoM cases (41.3%, P<0.001) than in other molecular subtypes (UPD 18.1%, IC1-GoM 28.6%) (Figure 1r). Polyhydramnios was more common among IC1-GoM patients (35.5%, P=0.016) than IC2-LoM (15.3%), UPD (14.9%) or CDKN1C variant (0%) cases (Figure 1s). We observed no difference in the occurrence of hypoglycemic (Figure 1t). Three patients deceased (1 IC2-LoM, 1 UPD, 1 IC1-GoM) of prematurity-related complications (two cases of sepsis consequent to urinary tract infection owing to ureteral malformations, one of respiratory insufficiency). Concerning cancer occurrence, 33 patients developed a neoplasm during their follow-up, which lasted on average 9.8±7.3 (median 8.9) years (age range 0–2 years n=67, 2–4 years n=56, 4–8 years n=75, >8 years n=120). Twenty-four malignant neoplasms were reported in 23 patients (7.2%) (Figure 1p) and 14 benign tumors (Figure 1q) were observed in 14 cases of which 3 also had a malignancy. No tumor was recorded in CDKN1C variant patients, whereas the incidence of malignant neoplasms varied significantly in the other three subgroups: 2.1% in IC2-LoM, 14.9% in UPD, and 25.8% in IC1-GoM patients (P<0.001). Wilms' tumor developed only in patients with IC1-GoM or UPD, being clearly the prevalent cancer in IC1-GoM patients (P<0.001) (Tab. 1). Hepatoblastoma was the most common tumor among UPD patients and was not

reported in the other molecular subgroups (P=0.003). The tumor-free probability curves according to the molecular defects are depicted in Figure 3. Age at tumor diagnosis in IC1-GoM, UPD, and IC2-LoM patients was 13.8±9.3, 19.1±18.6, and 13.6±3.2 months, respectively. Mean age at the diagnosis for Wilms' tumor, hepatoblastoma, and neuroblastoma was 18.6±13.0, 16.2±26.9, and 16.0±8.2 months. There was a significant difference in the incidence of benign tumors (P=0.009), which were increasingly more common in IC2-LoM, UPD, and IC1-GoM patients (Figure 1s). The histotypes seen in the molecular subgroups are reported in Table 1. Correlations between each of the BWS features were explored in the cohort. We found significant association between malignant neoplasms and hemihyperplasia (P<0.001) and organ enlargement (P=0.030), Wilms' tumor and hemihyperplasia (P=0.024), hepatoblastoma and hemihyperplasia (P=0.019), polyhydramnios and ureteral anomalies (P=0.017), nevus flammeus and ear malformations (P<0.001), organomegaly and abdominal wall defects (P=0.038), umbilical hernia (P=0.039), and diastasis recti (P=0.018). Fourteen among Wilms' tumor, hepatoblastoma, and pancreatoblastoma cases occurred in enlarged organs.

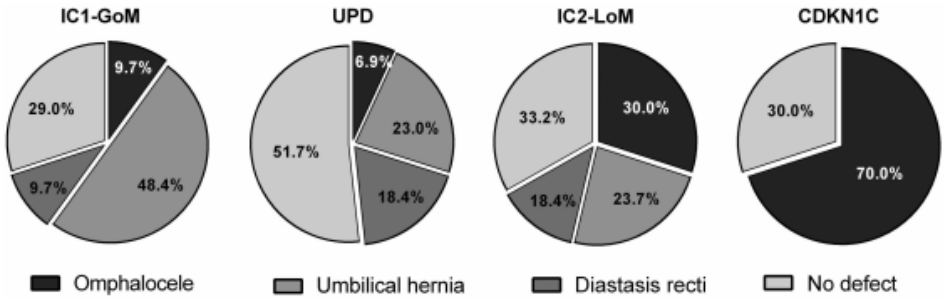


Figure 2 Enrichment of the abdominal wall defects in the molecular groups.

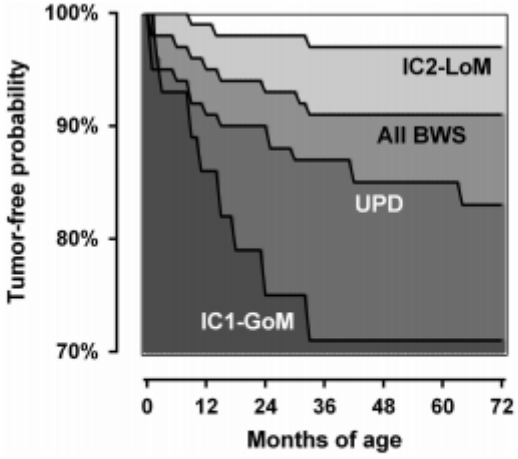


Figure 3 Kaplan–Meier plot of the tumor-free interval (malignant neoplasms only) in the three main molecular subtypes of Beckwith–Wiedemann syndrome (BWS). IC2-LoM: imprinting center 2 loss of methylation, UPD: paternal uniparental disomy; IC1-GoM: imprinting center 1 gain of methylation.

Table 1 Summary of the neoplasms reported

	Overall		*IC2-LoM*		*UPD*		*IC1-GoM*		*P*-value
	n	%	n	%	n	%	n	%	
Malignant tumors	25[a]	7.5%	4	2.1%	13	14.9%	8	25.8	<0.001
Wilms' tumor	10	3.1%	–	–	3	3.4%	7	22.6%	<0.001
Hepatoblastoma	5	1.6%	–	–	5	5.7%	–	–	0.003
Neuroblastoma	4	1.3%	2	1.1%	2	2.3%	–	–	
Pancreatoblastoma	2	0.6%	–	–	1	1.1%	1	3.2%	
Adrenal carcinoma	1	0.3%	–	–	1	1.1%	–	–	
Rhabdomyosarcoma	1	0.3%	1	0.5%	–	–	–	–	
Hemangiotelioma	1	0.3%	–	–	1	1.1%	–	–	
Germinoma	1	0.3%	1	0.5%	–	–	–	–	
Benign tumors	14	4.4%	4	2.1%	6	6.9%	4	12.9%	0.009
Hepatic angioma	9	2.8%	4	2.1%	4	4.6%	1	3.2%	
Thyroid adenoma	1	0.3%	–	–	1	1.1%	–	–	
Mammary gland fibroma	1	0.3%	–	–	1	1.1%	–	–	
Lipoma	1	0.3%	–	–	–	–	1	3.2%	
Pilomatrixoma	1	0.3%	–	–	–	–	1	3.2%	
Cavernous hemangioma	1	0.3%	–	–	–	–	1	3.2%	

[a]Twenty-three patients: one patient with UPD developed a pancreatoblastoma and a neuroblastoma.

Discussion

BWS is characterized by one of the widest phenotypic spectra of syndromic developmental disorders, ranging from lethal to mild and incomplete forms. This highly variable phenotypic expression is paralleled at the molecular level by a complex heterogeneity of (epi)genetic defects at chromosome 11p15.5. Correlations between genotype and phenotype have been previously reported in other BWS cohorts [Rump et al. 2005, Cooper et al. 2005, Brioude et al. 2013, Ibrahim et al. 2014, Bliek et al.2004]. In particular, omphalocele, ear signs, and nevus flammeus

were associated with IC2 LoM or CDKN1C variants, hemihyperplasia with UPD, and Wilms' tumor with IC1-GoM or UPD [Rump et al. 2005, Cooper et al. 2005, Brioude et al. 2013, Ibrahim et al. 2014, Bliek et al.2004]. In this study we further investigated these correlations providing data on a large cohort of fully characterized BWS patients with 11p15 region molecular defects. Our analysis evidences in the four BWS molecular subtypes differences in the incidence of many phenotypic traits, such as growth pattern, prevalence and severity of abdominal wall defects, macrosomia, nevus flammeus, ear signs, renal malformations, ureteral anomalies, organ enlargement, polyhydramnios, cancer incidence, and histotypes.

This analysis allows to define phenotypic profiles that are characteristic of the different molecular subgroups. Patients with IC1-GoM are constantly macrosomic at birth and commonly present abdominal wall defects – usually minor – consistent with organ enlargement; approximately one-third has renal anomalies and ureteral malformations that correlate with higher occurrence of polyhydramnios. IC2-LoM patients show an excess of premature births. In contrast, neonatal macrosomia is much less represented in this group and they rather present postnatal overgrowth. It is important to underline that the prevalence of macrosomia in BWS cohorts depends on its definition; we opted for the permissive definition of neonatal weight >90th percentile as of more diffuse usage, already employed in the definition of BWS diagnostic criteria [Rump et al. 2005], and used to define the large-for-gestational-age newborn in our setting [Bertino et al. 2010]. We confirm the increased prevalence of omphalocele in IC2-LoM further show that they have lower incidence of organ enlargement, suggesting that wall defects are primarily caused by developmental anomalies of the abdominal wall rather than consequent to increased abdominal pressure; nevus flammeus and ear signs are also particularly frequent in IC2-LoM patients (about half of the cases).

As previously reported, UPD patients typically present with hemihyperplasia; most of them have no abdominal wall defect, the others usually display only minor ones; concerning the other BWS-associated features, they generally show an intermediate prevalence with respect to IC1-GoM and IC2-LoM, consistent with the extent of the molecular defect, which affects both domains of the 11p15.5 cluster. It is worth to mention that there are conflicting results concerning the existence of a correlation

between phenotype severity in UPD cases and the level of somatic mosaicism or the extent of the chromosomal isodisomy [Calvello et al. 2013, Smith et al. 2007, Itoh et al. 2000, Romanelli et al. 2011]. In this study, however, we evaluated only the presence and not the severity of the single BWS features and did not explore the two above-mentioned molecular factors. Moreover, we excluded only in a fraction of the UPD patients genome-wide UPD, a genetic phenomenon linked to a further increase in cancer risk [Kalish et al. 2013] and additional phenotypic features [Inbar-Feigenberg et al. 2013].

As concerns CDKN1C patients, a striking overlap with IC2-LoM phenotype was evident; they shared a similar growth pattern with low incidence of neonatal macrosomia and frequent occurrence of postnatal overgrowth, excess of preterm births, comparable proportion of ear signs and nevus flammeus, low prevalence of organ enlargement. Consistent with previous observations [Cooper et al. 2005, Brioude et al. 2013], CDKN1C patients were characterized by the highest prevalence of omphalocele and cleft palate. Moreover, we did not detect any case of hemihyperplasia in this group. However, conclusions on CDKN1C phenotype should be drawn cautiously given the small number of patients included. Moreover, as we sequenced CDKN1C gene in a subset of selected patients, our data are prone to be biased.

It is well know that BWS is more common among patients conceived by artificial reproduction technique [Halliday et al. 2004, Sutcliffe et al. 2006, Hiura et al. 2012]; we encountered a 4.4% prevalence of this phenomenon, confirming data from previous reports and showing a higher prevalence than that reported in the Italian population (1.7%) [Kupka et al. 2014].

As concerns tumor risk, the overall prevalence of cancer approximates 8%, consistent with other studies [Brioude et al. 2013]. It is well established that patients with telomeric defects (IC1-GoM/UPD) have a major risk of tumors, especially Wilms' tumor, whereas patients with defects of the centromeric domain (IC2-LoM/CDKN1C variant) have a lower risk [Rump et al. 2005, Cooper et al. 2005, Brioude et al. 2013]. Our data also point to a gradient of oncogenic risk between the three main molecular subgroups. At one end of the spectrum, patients with IC2-LoM have a very low risk

of tumors (<2%) and do not develop Wilms' tumors. At the other end of the spectrum, patients with IC1-GoM have a very high tumor risk (25%) and are particularly prone to Wilms' tumor development. Between the two groups, UPD patients show an intermediate oncogenic risk (15%) and can develop histotypes seen in both IC1-GoM and IC2-LoM cases; furthermore, UPD cases show a previously unreported predisposition to hepatoblastoma, the second more common histotype of BWS, occurring in 1.6% of BWS patients, that is, 6% of UPD cases. We did not observe hepatoblastoma in the other molecular subgroups, but cannot conclude that hepatoblastoma occurs only in UPD cases, as three cases have been described in IC2-LoM patients previously [Rump. P etal. 2005, Brioude F. et al. 2013].

Few data are available on benign neoplasms in BWS [Weksberg et al. 2001, Cohen 2005, Cappuccio et al. 2014]; interestingly, their incidence is a gradient across the molecular subtypes paralleling that of malignancies: highest (~13%) in IC1-GoM, intermediate (~7%) in UPD, and lower (~4%) in IC2-LoM patients. Among benign histotypes observed, hepatic angiomas were prevailing, and no differences were detectable across the molecular subgroups. Several of the correlations evidenced between (epi)genotype and phenotype consolidate previous observations (Table 2) [Rump et al. 2005, Cooper et al. 2005, Brioude et al. 2013, Ibrahim et al. 2014, Bliek et al. 2004,].

Table 2 Significant genotype–phenotype correlations in the three most largest recent correlation studies on BWS

Genotype	*Ibrahim, 2014 (n = 507)*[13]	*Brioude, 2013 (n = 407)*[10]	*This study (n = 318)*
IC2-LoM	*n* = 321 Macroglossia Omphalocele Ear malformations Nevus flammeus Malignant tumors 0.9% (1 Wilms' tumor, 1 hepatoblastoma, 1 rhabdomyosarcoma)	*n* = 257 Omphalocele Ear malformations Nevus flammeus Malignant tumors 3.1% (2 Neuroblastoma, 2 hepatoblastoma, 1 sarcoma, 1 rhabdomyosarcoma, 1 thyroid carcinoma, 1 melanoma)	*n* = 190 Macroglossia Postnatal overgrowth Omphalocele Ear malformations Nevus flammeus Preterm birth Malignant tumors 2.1% (2 neuroblastoma, 1 rhabdomyosarcoma, 1 germinoma)
UPD	*n* = 135 Hemihyperplasia Umbilica hernia Malignant tumors 6.7% (3 Wilms' tumor[a], 5 Hepatoblastoma, 1 adrenal cortical carcinoma)	*n* = 81 Hemihyperplasia Organ enlargement Hypoglycemia Malignant tumors 17.3% (10 Wilms' tumor, 2 adrenal cortical carcinoma, 2 hepatoblastoma, 1 rhabdomyosarcoma, 1 neuroblastoma, 1 acute lymphoid leukemia)	*n* = 87 Hemihyperplasia Umbilical hernia Renal anomalies Malignant tumors 14.9% (5 Hepatoblastoma, 3 Wilms' tumor, 2 neuroblastoma, 1 pancreatoblastoma, 1 hemangiotelioma, 1 adrenal cortical carcinoma)
IC1-GoM	*n* = 47 Diastasis recti Malignant tumors 8.5% (4 Wilms' tumor 1 hepatoblastoma[b])	*n* = 35 Neonatal overgrowth Hemihyperplasia Organ enlargement Hypoglycemia Malignant tumors 28.6% (10 Wilms' tumor)	*n* = 31 Neonatal overgrowth Macroglossia Diastasis recti Organ enlargement Renal and ureteral anomalies Polyhydramnios Benignant neoplasms Malignant tumors 25.8% (7 Wilms' tumor, 1 pancreatoblastoma)
CDKN1C variants	Not tested	*n* = 34 Omphalocele Ear malformations Nevus flammeus Malignant tumors 8.8% (1 neuroblastoma, 1 ganglioneuroma, 1 acute lymphoid leukemia)	*n* = 10 Postnatal overgrowth Omphalocele Ear malformations Nevus flammeus Preterm birth No tumors reported

[a]One patient with a 11p15.5 duplication was included in the UPD group for simplicity.
[b]The patient is reported to have been diagnosed with both Wilms' tumor and hepatoblastoma.

Some aspects emerge as new: in particular, the significant association between hepatoblastoma and UPD may have relevant implications for cancer screening, the association between IC2-GoM and uretheral defects and polyhydramnios may have implications for the neonatal nephrourological management, the higher incidence of benign neoplasm paralleling the distribution of the malignant ones should be taken into considerations during patients' follow-up. Finally, IC2-LoM/CDKN1C variant patients display a higher rate of postnatal overgrowth, poorly studied before; as in these molecular subgroups neonatal macrosomia is rarer than in UPD/IC1-GoM ones [Ibrahim et al. 2014] clinicians should be aware that these molecular subtypes of BWS may display specific growth patterns after the neonatal period. Based on these and recent findings [Rump et al. 2005, Brioude et al. 2014], we suggest a revision of the guidelines for tumor surveillance that takes into consideration the molecular defects. At present, cancer surveillance programs for BWS patients are based on a 3–6 months abdominal ultrasound up to 7–8 years of age to detect Wilms' tumor and 2–3 months serum alpha-fetoprotein determinations up to 4 years of age to screen for hepatoblastoma [Rump et al. 2005].

Cost-effectiveness of ultrasound screening is proven [McNeil et al. 2001]; we hypothesize that patients with IC1-GoM may benefit from an intensification of abdominal ultrasound during the first 3 years of life, as most of Wilms' tumors are diagnosed before that age and appears justified by their 25% chance of developing a Wilms' tumor, the well-proven beneficial effect of early diagnosis, and the low invasivity of abdominal ultrasound [Scott et al. 2006]. Conversely, the dosage of the tumor marker serum alpha-fetoprotein as a screening method for the early diagnosis of hepatoblastoma is debated, given the complexity of its interpretation in childhood [Mussa et al. 2014, Mussa et al. 2011], the low incidence of hepatoblastoma and the invasivity of frequent blood drawns, which is commonly responsible for the lack of adherence to screening protocols [Zarate et al. 2009]. We believe that monitoring is worthwhile at least in UPD patients, given their high risk of hepatoblastoma. More questionable is the employment of ultrasound and tumor markers screening in IC2-LoM cases, given the low risk and the occurrence of histotypes for which the advantage of these screening methods is still unproven. In this molecular group, which represents >50% of BWS cases, clinical research should be focused in assessing its cost-effectiveness and further studies are needed to assess its ability to detect IC2-LoM-related malignancies and the actual impact of the detection timing on their management and treatment. A clinical follow-up with ad hoc instrumental/laboratory investigations may prove to be a reasonable alternative.

In conclusion, although none of the BWS phenotypes can be considered specific to a molecular anomaly, the relevant differences observed in the four molecular subtypes allow to speculate that BWS could be separated into four different conditions with different malformative pattern and specific phenotypic profile despite some degree of clinical overlap. This composite view of this syndrome likely has relevant implications and does impact on clinical care of patients allowing to move toward a (epi)genotype-based follow-up.

Chapter 2.

Defining the fetal growth patterns in Beckwith- Wiedemann syndrome

This work has been published as

Mussa A, Russo S, de Crescenzo A, Freschi A, Calzari L, Maitz S, Macchiaiolo M, Molinatto C, Baldassarre G, Mariani M, Tarani L, Bedeschi MF, Milani D, Melis D, Bartuli A, Cubellis MV, Selicorni A, Silengo MC, Larizza L, Riccio A, Ferrero GB. **Fetal growth patterns in Beckwith-Wiedemann syndrome**. Clin Genet. 2016 Jul;90(1):21-7.

BACKGROUND

Few data are currently available on fetal and neonatal growth in BWS, being cancer predisposition the main focus of the available clinical reports given the implications for tumor screening. Data on growth pattern are largely insufficient: previous reports sought a widely variable prevalence of fetal and neonatal macrosomia in affected cohorts, probably reflecting collection biases, different prevalence of the molecular anomalies, and variable definition of growth excess. So we systematically analyzed the neonatal anthropometric data of a large cohort of molecularly confirmed BWS patients to look for (epi)genotype/phenotype correlations in order to highlight the phenotypic differences, to deepen the pathogenetic mechanism of the disease, and to develop clinical protocols based on the molecular diagnosis.

Materials and methods

Patients

Data from 247 BWS patients were collected through a national search in two laboratories providing genetic testing in Italy and through the involvement of the major clinical genetics centers of the country. Of those, 231 were also included in a previous report from our group [Mussa et al. 2016]. Clinical information and data concerning pregnancy, fetal development, birth and neonatal measurements were collected directly by the physicians who made the diagnosis. Data were collected only from patients with at least two diagnostic clinical criteria for BWS diagnosis (among abdominal wall defects, macroglossia, macrosomia, embryonal tumor, ear malformations, organ enlargement, *naevus flammeus*, hemihyperplasia, nephrourological urinary tract malformations, cleft palate, hypoglycemia, family history of BWS) and proven molecular defects were included: cases with negative molecular tests were excluded. Only singleton pregnancies were included.

Genotyping

DNA was extracted from peripheral blood lymphocytes by standard procedures. Methylation analysis of the 11p15.5 chromosomal region including IC1 and IC2 was carried out either by Southern blotting (n = 98), combined bisulfite restriction analysis (COBRA) (n = 57) [Alders et al. 2009] or Methylation-Sensitive Multiple Ligation Probe Amplification (MS-MLPA kit, MRC-HOLLAND, Amsterdam, The Netherlands)

(*n* = 98) [Priolo et al. 2008]. The results obtained by these different techniques are comparable [Priolo et al. 2008, Bliek et al. 2009]. In patients with suspected UPD, microsatellite analysis was carried out as reported [Russo et al. 2003]. *CDKN1C* gene sequencing was performed as described elsewhere [Romanelli et al. 2010] in cases without 11p15.5 methylation anomalies and with affected relatives, palatoschisis, or major/recurrent abdominal wall defects. All patients or their parents provided written informed consent.

Definitions

Neonatal growth was standardized according to gestational age- and sex-corrected population-specific standards for newborns between 23 and 42 weeks of gestation [Bertino et al. 2010], and expressed as standard deviation score (SDS). Neonatal weight, length and head circumference were gathered from delivery clinical records and measured according to guidelines [Bertino et al. 2012]. Large for gestational age (LGA), implying overgrowth, was defined as birth weight exceeding the 90th percentile for gestational age [Bertino et al. 2012], small for gestational age (SGA) as weight <10th percentile, and adequate for gestational age (AGA) as weight between the 10th and 90th percentiles. Prematurity was defined as birth <37 weeks of estimated gestational age. To assess the relationship between body weight and length, three proportionality indexes have been employed: (i) Quetelet index (QI), as the ratio between the two variables (weight/length, expressed in g/cm); (ii) Kaup index (KI), as the ratio between weight × 10 and the square of the length (weight × 10/lenght^2, expressed in g/cm^2); (iii) ponderal index (PI), as the ratio between weight × 100 and the third power of the length (weight × 100/lenght^3, expressed in g/cm^3) [Song et al. 2013].

Comparisons among the molecular subtypes have been conducted by Fisher's exact or χ^2tests with Yates correction for continuity, as appropriate. Comparisons between continuous variables (SDS) were conducted by one-way analysis of variance (ANOVA) with Bonferroni's *post hoc* group test. Comparison of neonatal SDS with reference values was conducted by one sample Student's *t*-test employing *zero* as mean value. Two-tailed p values were considered as significant when <0.05. Data have been analyzed by Prism Graph Pad 5.0 (La Jolla, CA).

Results

Of the 247 BWS patients included 21 (8.5%) harbor IC1-GoM, 68 (27.5%) UPD, 147 (59.5%) IC2-LoM (5 patients from 2 families with duplication and 1 with a deletion involving the IC2) and 11 (4.5%) *CDKN1C* mutations. The four molecular subgroups were distinguishable by different mean gestational ages, lowest in IC1-GoM and *CDKN1C* cases, intermediate in IC2-LoM ones and higher in UPD ones ($p < 0.001$). In the *CDKN1C* mutation group, the proportion of preterm birth was the highest (63.3%), followed by that of patients of the IC2-LoM group, with respect to that observed in the other groups ($p = 0.004$). Moreover, the subgroups displayed significant differences in the prevalence of cases classifiable as LGA ($p = 0.026$) or AGA ($p < 0.001$) (Table 1): 95.2 % IC1-GoM cases were LGA while 63–69% LGA cases were present in the other molecular group.

Table 1. Neonatal weight in the four Beckwith–Wiedemann molecular subgroups

	Mean gestational age ± SD	Preterm (<37 g.w.)	LGA	AGA	SGA
IC1-GoM	36.3 ± 3.6	6 (28.6%)	20 (95.2%)	1 (4.8%)	0
UPD	38.1 ± 1.6	12 (17.6%)	47 (69.1%)	20 (29.4%)	1 (1.5%)
IC2-LoM	37.2 ± 2.5	54 (36.7%)	96 (65.3%)	47 (32.0%)	4 (2.7%)
CDKN1C	37.0 ± 3.2	7 (63.6%)	7 (63.3%)	4 (36.7%)	0
Total	37.3 ± 2.5	79 (32.0%)	170 (68.8%)	72 (29.1%)	5 (2.1%)
p	<0.001	0.004	0.026	<0.001	n/a

AGA, adequate for gestational age; CDKN1C, cyclin-dependent kinase inhibitor 1C gene; IC1-GoM, imprinting center 1 gain of methylation; IC2-LoM, imprinting center 2 loss of methylation; LGA, large for gestational age; n/a, not available; g.w., gestational weeks; SGA, small for gestational age; UPD, paternal uniparental disomy for chromosome 11p15.5.

The plot of neonatal parameters in the molecular subgroups is depicted in Fig. 1.

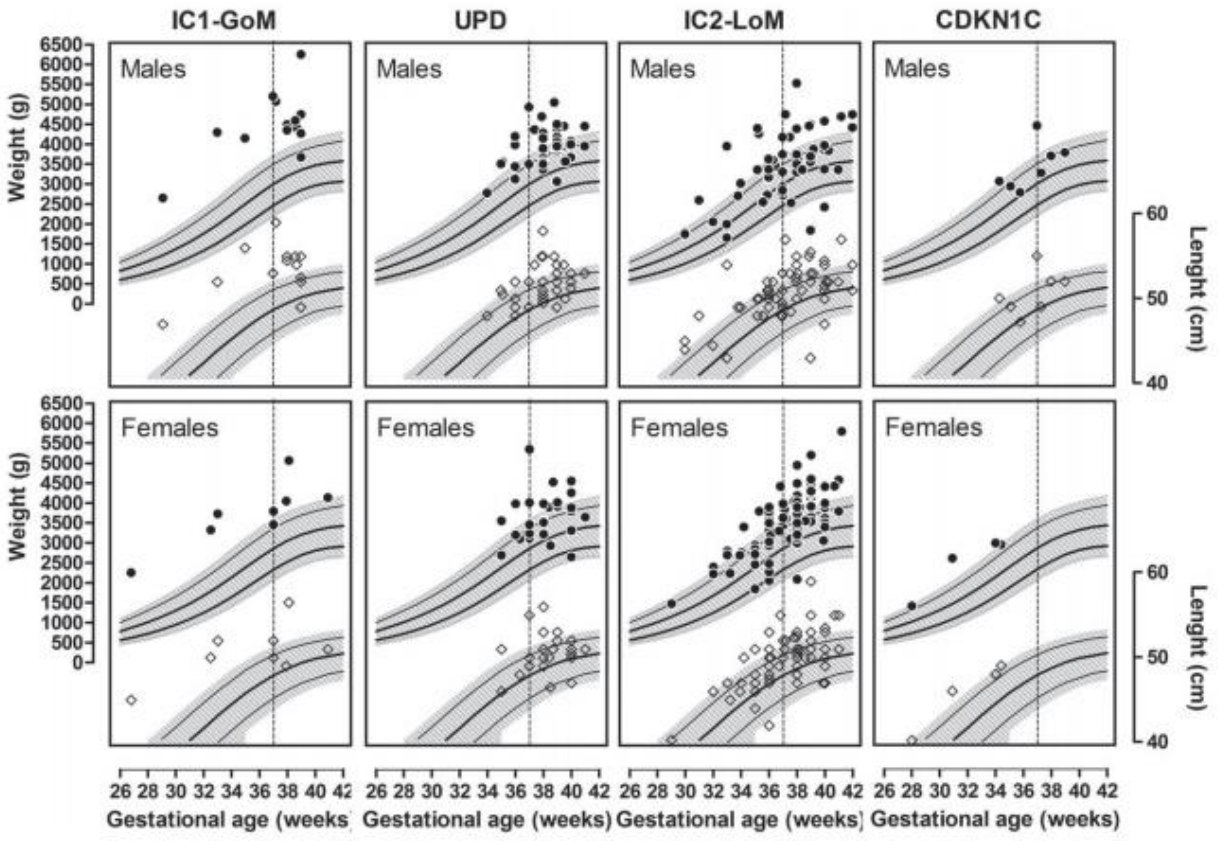


Fig. 1. Neonatal weight (filled circles) and length (empty diamonds) in the molecular subgroups of Beckwith–Wiedemann patients plotted against respective gestational age. Shaded areas cover the 3rd–97th percentile interval and the 10th, 50th and 90th percentiles are represent with black lines. The vertical dashed line divides preterm and term babies according to the 37 weeks cut-off. IC1-GoM, imprinting center 1 gain of methylation; IC2-LoM, imprinting center 2 loss of methylation; UPD, uniparental disomy.

As concerns neonatal measurements, all the four molecular subgroups had weight SDS and length SDS significantly higher than normal ($p < 0.001$), 4.41 ± 2.04 and 2.93 ± 1.60 SDS in IC1-GoM patients, 2.04 ± 1.44 and 1.38 ± 1.36 SDS in UPD patients, 1.78 ± 1.64 and 1.51 ± 1.60 SDS in IC2-LoM patients, and 1.95 ± 1.26 and 1.66 ± 1.10 SDS in CDKN1C patients, respectively. Head circumference SDS were significantly higher than normal in IC1-GoM, IC2-LoM and CDKN1C mutation patients (1.59 ± 1.72 SDS $p = 0.0012$, 1.00 ± 1.33 SDS $p < 0.001$, and 0.64 ± 0.78 SDS $p = 0.027$, respectively), but not in UPD cases (0.80 ± 1.12 SDS). Weight and length but not head circumference SDS of patients with IC1-GoM were higher than those of the UPD patients, and IC2-LoM and CDKN1C mutation groups, which did not significantly differ for weight and length one to each other (Fig. 2a–c).

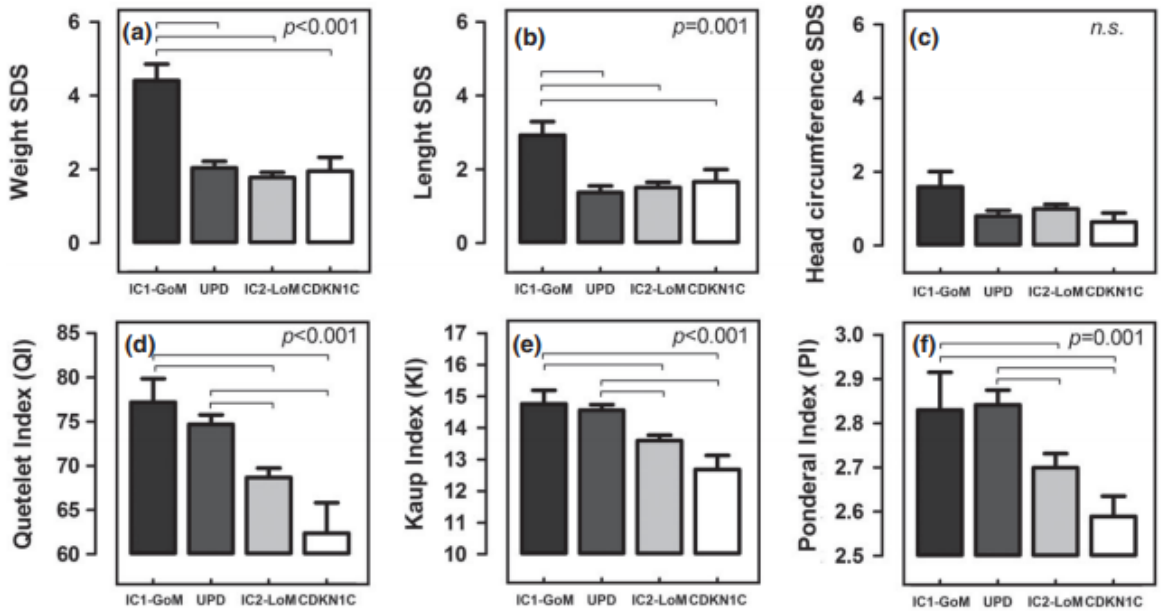


Fig. 2. Standard deviation scores (SDS) of neonatal weight (**a**), length (**b**), and head circumference (**c**), and of the three mass indexes of Quetelet (QI = weight/length, (**d**), Kaup (KI = weight × 10/lenght^2, (**e**), and Ponderal index (PI = weight×100/lenght^3, (**f**) in the four molecular subgroups of Beckwith–Wiedemann syndrome (BWS). Imprinting center 1 gain of methylation cases (IC1-GoM) are in black, paternal uniparental disomy for chromosome 11p15.5 (UPD) cases are in dark gray, imprinting center 2 loss of methylation (IC2-LoM) cases in light gray, and cases with CDKN1C mutation in white. P values in the topright corner of each panel refer to one-way ANOVA (n.s., not significant), horizontal lines represent significant ($p < 0.05$) differences between each molecular group at Bonferroni *post hoc* test.

The comparison of the proportionality indexes revealed that the ratio between weight and length (QI) was different across the molecular groups depicting a gradient: IC1-GoM patients had the highest index (77.2 ± 11.8 g/cm), followed by the UPD patients (74.7 ± 8.4 g/cm), IC2-LoM (68.7 ± 12.1 g/cm) and CDKN1C mutation ones (62.3 ± 11.5 g/cm) (Fig. 2d). The two other mass indexes (KI and PI) were similar in IC1-GoM and UPD cases (KI 14.8 ± 1.9 vs 14.6 ± 1.4 and PI 2.83 ± 0.37 vs 2.84 ± 0.26, respectively) but significantly lower in IC2-LoM and CDKN1C ones (KI 13.6 ± 2.0 vs 12.6 ± 1.5 and PI 2.70 ± 0.35 vs 2.59 ± 0.15, respectively) (Fig. 2e,f). No differences were present between the two sexes or between patients with and without neoplasms.

A correlation analysis between neonatal SDS and gestational ages identified further differences among the molecular subtypes of BWS. In IC1-GoM patients, the gestational age was negatively correlated with weight and length excess, positively with QI and KI. In UPD cases, only the weight SDS was negatively correlated with gestational age, but not with other parameters. In IC2-LoM, the negative correlation was evident only with head circumference SDS, whereas the positive one was found with all the three mass indexes measured. No correlation was found for the cases with CDKN1C mutation (Table 2).

Table 2. Correlations between gestational age and the anthropometric neonatal parameters in the four Beckwith–Wiedemann molecular subgroups (r = correlation coefficient, p = correlation significance, n.s., not significant)

	Weight SDS	Length SDS	Head circumference SDS	QI	KI	PI
IC1-GoM	$r = -0.567$ $p = 0.007$	$r = -0.623$ $p = 0.005$	n.s.	$r = +0.738$ $p < 0.001$	$r = +0.648$ $p = 0.003$	$r = +0.532$ $p = 0.021$
UPD	$r = -0.257$ $p = 0.034$	n.s.	n.s.	n.s.	n.s.	n.s.
IC2-LoM	n.s	n.s.	$r = -0.206$ $p = 0.024$	$r = +0.601$ $p < 0.001$	$r = +0.526$ $p < 0.001$	$r = +0.346$ $p < 0.001$
CDKN1C	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Overall	$r = -0.176$ $p = 0.006$	$r = -0.192$ $p = 0.005$	$r = -0.245$ $p < 0.001$	$r = +0.530$ $p < 0.001$	$r = +0.469$ $p < 0.001$	$r = +0.309$ $p < 0.001$

AGA, adequate for gestational age; CDKN1C, cyclin-dependent kinase inhibitor 1C gene; IC1-GoM, imprinting center 1 gain of methylation; IC2-LoM, imprinting center 2 loss of methylation; LGA, large for gestational age; n/a, not available; g.w., gestational weeks; SGA, small for gestational age; UPD, paternal uniparental disomy for chromosome 11p15.5.

Discussion

The data collected in our BWS cohort demonstrate that the four molecular subgroups of BWS patients are characterized by specific fetal growth patterns. A gradient in neonatal overgrowth is displayed across the subgroups, with weight and length excess decreasing progressively from IC1-GoM cases to IC2-LoM/CDKN1C ones and UPD cases displaying intermediate auxometric patterns. The proportion of LGA cases varies accordingly, with almost all IC1-GoM patients being scored as LGA compared to approximately two thirds in the other molecular groups. Our data confirm on a larger scale previous investigations reporting higher neonatal weight in BWS patients with IC1-GoM defect [Cooper et al. 2005, Mussa et al. 2016]. The trend observed for neonatal weight excess across the molecular subgroups is an intriguing phenomenon which has also been observed by Brioude et al. [Brioude et al. 2013]: the SDS of the parameters measured in our series are substantially consistent with those reported in the above study, with the exception of the IC1-GoM cases, which in our cohort appear to have higher weight and length.

It is evident that a subset of BWS patients not only shows increased neonatal anthropometric parameters but also displays a disproportion between weight and length excesses. The relationship between these parameters was investigated by three indexes commonly used in clinical practice (QI, KI, PI) to estimate the ratio between neonatal weight and length. The magnitude of the disproportion between these parameters is different across the four molecular subgroups, displaying a distinctive pattern. IC1-GoM cases show the highest degree of disproportion between weight and length, both in excess, with the former largely prevailing. An intermediate degree of disproportion was

observed in UPD cases, whereas neonatal indexes were almost normal in the IC2-LoM/CDKN1C cases, indicating that the growth excess impairs almost equally weight and length in such cases. The distinction between proportional/disproportional somatic growth pattern also defined as 'symmetrical/asymmetrical' intrauterine growth is widely employed to describe syndromic and non-syndromic conditions with fetal growth restriction. Parallely, this definition could be applied to the overgrowth context defining IC1-GoM and UPD cases as 'asymmetrically LGA'. Disproportion between measurements was previously observed also prenatally [Kagan et al. 2015].

Premature birth in BWS is often described as a random event or as a complication of the malformations of the BWS spectrum. In our cohort, the prevalence of prematurity is 25% and although it can occur in each of the molecular groups, it prominently affects CDKN1C and IC2-LoM patients. In CDKN1C patients, prematurity has been observed in 64% of cases, whereas in the IC2-LoM group it occurs in 37% of cases. As IC2-LoM is the most common epigenetic defect, accounting for more than 50% of cases, prematurely born IC2-LoM patients overall represent more than three quarters of the premature births seen in this syndrome.

It is also interesting to note the specific somatic developmental parameters and their modifications during the progression of pregnancy across the subgroups: weight and length excess decreases with the progression of gestational age in IC1-GoM cases, but it remains constant in IC2-LoM cases. The interpretation of this phenomenon is complex and may lead to speculate that the timing of gene expression of the two domains is regulated differently during the embryogenesis and the fetal life.

The differences in neonatal growth pattern observed across the four molecular subgroups likely reflect differences in the regulation and function within the 11p15.5 imprinted gene cluster [Cordeiro et al. 2014]. The two different methylation anomalies of BWS (IC1-GoM and IC2-LoM) appear at the two end of the spectrum, while UPD cases display an intermediate growth pattern, possibly reflecting the degree of somatic mosaicism. The pronounced severity of IC1-GoM phenotype probably depends on a more pronounced gene deregulation with respect to that of IC2-LoM. GoM at IC1 causes both biallelic expression of the growth factor IGF2 and reduced expression of the tumor suppressor H19, providing a cooperatively additive mechanism promoting cell proliferation [Sun et al. 1997, Choufani et al. 2010].

IGF2 is a well-known direct regulator of growth during fetal development and early life. Its tissutal action has been extensively studied in both mouse models of BWS and affected humans. The degree of expression of IGF2 correlates with the degree of fetal hyperplasia of the various tissue affected in BWS [Hedborg et al. 1994]. Observations indicating an acceleration of BWS overgrowth during the second trimester of pregnancy are confirmed by our data showing a progression of the auxometric indexes with gestational age [Angiolini et al. 2011, Caspary et al. 1999].

The neonatal anthropometric phenotypes of IC2-LoM and CDKN1C cases are very similar, consistent with the common reduction in CDKN1C activity. Indeed, IC2-LoM is associated with CDKN1C downregulation [Diaz-Meyer et al. 2003]. Consistent with the human phenotype, the Cdkn1c knock-out mouse show some phenotypic traits of BWS but does not exhibit pronounced overgrowth [Tunster et al. 2011]. Fetal growth in murine models is characterized by early moderate weight excess (approximately 20%) but subsequent growth trajectory reversal, likely caused by progressive loss of the integrity of the placental interface due to sinusoid thrombotic lesions [Romanelli et al 2009]. It has been proposed that CDKN1C loss of function (LoF) mutations have a role in the development of pregnancy-related complications, specifically preeclampsia/HELLP (hemolysis, elevated liver enzymes, low platelet count) syndrome [Romanelli et al. 2009]. In pregnancies of CDKN1C mutant human [Romanelli et al. 2009] and mice fetuses, abnormal placental development, premature labor, increased blood pressure, proteinuria and [Kanayama et al. 2002] renal lesions are observed; these complications are caused by the fetoplacental unit and not by the maternal genetic defect. Our data are consistent with the observations in animal models demonstrating in CDKN1C mutant and IC2-LoM cases an excess of prematurity but a growth excess milder than that observed in IC1-GoM/UPD cases. Consistently, correlation between the degree of fetal overgrowth and gestational age was observed in IC1-GoM/UPD but not in IC2-LoM/CDKN1C cases.

Different degree of mosaicism of UPD, IC1-GoM and IC2-LoM that have not been explored in this study may cause further variability in body size. Additional factors, such as nutrition, folic acid assumption, maternal or fetal pregnancy complications may also unpredictably influence the growth patterns and duration of gestation of BWS patients.

In conclusion, BWS is characterized by a clear and intriguing correlation between 11p15.5 (epi)genotype and fetal growth phenotype. IC1-GoM cases almost constantly show extreme neonatal macrosomia and severe disproportion between weight and length, representing a specific form of asymmetrical LGA. In IC2-LoM/CDKN1C cases, macrosomia is less common at birth and usually of a more modest entity with respect to IC1-GoM cases. Newborns with IC2-LoM/CDKN1C defects are usually proportionate, as weight and length are almost equally affected. Moreover, IC2-LoM patients display an excess of preterm births which is relevant to take into account, as this group overall represents more than half of BWS cases. Patients with UPD show neonatal weight and LGA fraction closer to those of IC2-LoM, but manifest a body mass disproportion rather similar to that seen in IC1-GoM cases. CDKN1C cases seem to share most anthropometric measurements with IC2-LoM patients, consistent with similar molecular etiology. This group also displays the highest percentage of prematurity. As concerns the perinatal management and clinical practice implications, the results of this research allow to estimate that most of the preterm BWS newborns have IC2-LoM—approximately 70%—and that contrarily to expectations half of BWS has normal or only borderline-high birth parameters. Therefore, clinicians should have a low diagnostic suspect threshold for BWS also in absence of neonatal overgrowth. The differences observed in the neonatal indexes, besides providing interesting clues in the mechanisms of fetal growth, also allow to hypothesize that to the different molecular defects of BWS may correspond different growth patterns in childhood and different metabolic implications later in life which need to be addressed in future research.

As a final point, fetal growth patterns allow to separate BWS in different phenotypic profiles based on (epi)genotype, and growth trajectories are remarkably consistent with the gene deregulation primed by the different molecular defects.

Chapter 3. A novel (Epi)Genotype specific histotype targeted screening protocol: defining cancer risk in Beckwith-Wiedemann syndrome

This work has been published as

Mussa A, Molinatto C, Baldassarre G, Riberi E, Russo S, Larizza L, Riccio A, Ferrero GB. **Cancer Risk in Beckwith-Wiedemann Syndrome: A Systematic Review and Meta-Analysis Outlining a Novel (Epi)Genotype Specific Histotype Targeted Screening Protocol**. J Pediatr. 2016 Sep;176:142-149

BACKGROUND

Certainly cancer risk is the most concerning issue for the families of BWS patients and it is also the most expensive aspect of the clinical management. Specific tumor-surveillance procedures are used for the early detection of the 2 most commonly observed histotypes: Wilms tumor and hepatoblastoma [Rump et al. 2003]. Tumor screening consists in repeated abdominal ultrasound scanning (every 3-4 months during the first 8-10 years of life) and serum alpha-fetoprotein (αFP) measurement (every 2-3 months during the first 4 years of life) [Rump et al. 2003, Lapunzina 2005, Mussa et al. 2016].

The 4 main molecular subtypes of BWS (ICR2-LoM, ICR1-GoM, UPD, and CDKN1C mutation) are characterized by specific genotype-phenotype correlations, including tumor risk.3, 9, 10, 11 It seems that tumor predisposition results primarily from dysregulation at the telomeric domain of 11p15 (ICR1-GoM and UPD) rather than at the centromeric one (ICR2-LoM and CDKN1C mutation) [Rump et al. 2003]. In addition, reports suggest that the molecular subtypes of the syndrome predispose to the development of different tumor histotypes [Ibrahim et al. 2014, Brioude et al. 2013, Mussa et al. 2015].

The ability to identify patients with the greatest tumor risk is important for optimal management and counseling. A deeper knowledge of the type and incidence of embryonal tumor to which different molecular lesions predispose may allow more refined surveillance protocols and personalized strategies for tumor screening. The aim of this study was to combine the information available from the literature and meta-analyze the results to further strengthen (epi)genotype-cancer risk correlations and to allow meaningful statistical comparisons between BWS molecular subtypes.

Methods

We collected data from the literature in November 2015 via PubMed, the Cochrane Library, and Scopus, searching for original research articles on BWS and tumor development with no language restriction. References cited in reviews and original papers also were examined. The keywords used were “Beckwith-Wiedemann

syndrome" and "Wiedemann-Beckwith syndrome"; the search was limited to the last 15 years (January 2000 through November 2015).

Only studies that provided information on genotype-phenotype correlations between the specific molecular subtypes of BWS (ICR2-LoM, ICR1-GoM, UPD, CDKN1C mutation) and tumor frequency and specific histotypes were included in the meta-analysis. Case reports and small case series (n < 10), duplicate series, review articles, and articles with cohorts with other molecular defects (eg, multiple methylation defects) were excluded to avoid selection biases. Only studies with at least the following data were collected and tabulated: patient numbers, patients in each molecular subgroup analyzed, and patients with cancer in each molecular subgroup and histotypes detected. In studies lacking relevant data or potentially overlapping ones the authors were contacted.

Three investigators searched the literature independently, reviewed, and assessed the articles based on the review protocol. No abstracts of unpublished studies were found, including a search in the Google search engine. A search of publications retrieved 834 results; we excluded 817 studies after we screened them by reading the title or abstract, and we assessed the remaining 17 entries for eligibility by reading the full text. Ten were excluded because of insufficient data, nonpertinent information, or cohort overlap, confirmed by contact with the authors of the publications. Seven studies were judged adequate for the purpose of the study: Table I (available at www.jpeds.com) [Ibrahim et al. 2014, Brioude et al. 2013, Mussa et al. 2015, Brioude et al. 2015, Sasaki et al. 2007, Bliek et al. 2004, Weksberg et al. 2001, Vals et al. 2015, Calvello et al. 2013, Mussa et al. 2012, Zarate et al. 2009, Cooper et al. 2005 DeBaun et al. 2002, Goldman et al. 2002, Bliek et al. 2001, Gaston et al. 2001, Engel et al. 2000] displays the details of the 17 studies screened for eligibility. Article selection is described in Figure 1 (available at www.jpeds.com). All studies included were observational.

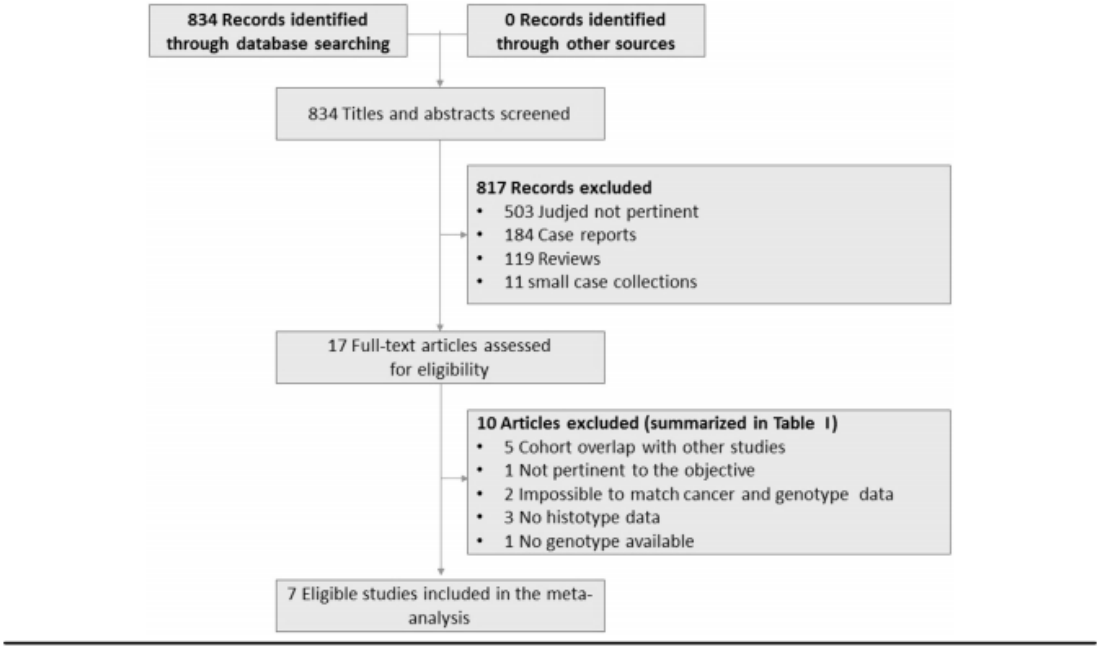


Figure 1. Flow diagram of study selection.

Table I. Summary of the relevant studies retrieved

Authors	Year	Cohort size, n	Cancer patients, n (%)	ICR1-LoM, n	UPD, n	ICR1-GoM, n	*CDKN1C* mutation, n	Included in meta-analysis	
Summary of studies included in the meta-analysis								**Age at follow-up, mean ± SD, y**	**Age at tumor diagnosis, mean ± SD**
Brioude et al[12]	2015	50	3 (6.0%)				50*	-	14.9 ± 18.3 y*
Mussa et al[11]	2015	318	24 (7.5%)	190	87	31	10	9.8 ± 7.3 y	15.5 ± 15.2 mo
Ibrahim et al[9]	2014	507	17 (3.4%)	321	135	47	Not tested	-	3.7 y
Brioude et al[10]	2013	407	37 (9.1%)	257	81	35	34*	-	28.4 ± 32.2 m
Sasaki et al[13]	2007	26	4 (15.4%)	15	7	0	4	-	-
Bliek et al[14],†	2004	48	6 (12.5%)	27	13	7	1	>5 y	-
Weksberg et al[15]	2001	81	12 (14.8%)	35	21	3	5	-	-
Summary of studies excluded from the meta-analysis								**Reason for exclusion**	
Vals et al[16]	2015	1	0	1				No, case report	
Calvello et al[17]	2013	19	7 (36.8%)	4	8	7		Overlaps Mussa et al[11]	
Mussa et al[18]	2012	42	2 (4.8%)	25	11	6	Not tested	Overlaps Mussa et al[11]	
Zarate et al[19]	2009							Genotyping not performed	
Cooper et al[20]	2005	200	8 (4.0%)	116	54	14	16	Overlaps Ibrahim et al[9]	
DeBaum et al[21]	2002	58	1 (1.1%)	39	9	10	Not tested	No histotype data, impossible to match cancer data with genotype	
Goldman et al[22]	2002	51	-	22	21	Not tested	8	Impossible to match cancer data with genotype	
Bliek et al[23]	2001	46	5 (10.9%)	31	11	4		Overlaps Bliek et al[14]	
Gaston et al[24]	2001	97	9 (9.3%)	45	11	11	2	Overlaps Brioude et al[3]	
Engel et al[25]	2000	70	0 (0.0%)	35	22	5	13	No histotype data, overlaps Ibrahim et al[9]	

*Patients with *CDKN1C* mutations overlapping those from Brioude et al[10] (included the series from Brioude et al[12]).
†Excluded data from the French cohort overlapping Gaston et al.[24]

Statistical Analyses

Two-way contingency tables were constructed on the basis of reported frequency distributions of tumors for each molecular subgroup. Data were analyzed statistically by Graph Pad 6.0 (GraphPad Software Inc, La Jolla, California) by the use of $\chi 2$ or Fisher

tests and assuming as statistically significant results with P < .05. Meta-analysis was performed by OpenMeta[Analyst] cross-platform software for advanced meta-analysis for Windows 8 (http://www.cebm.brown.edu/open_meta/) with consideration of tumor/histotype development as outcome and the molecular subgroup as exposure and by calculation of: (1) the tumor development OR of patients with ICR1-GoM, UPD, and CDKN1C mutations compared with the tumor ratio observed in cases of ICR2-LoM (selected as comparison group as known to be the group with the lowest tumor risk); and (2) all the histotypes associated to a specific molecular subgroup in the pooled analysis the OR of that histotype compared with any of the other subgroups taken singularly and grouped together. Only significant results are shown. Meta-analysis were performed with a random-effect model, and results are shown as ORs and corresponding 95% CIs and relative weight (presented for individual studies and pooled results) and also P values and I2 percentage of variation across studies for heterogeneity for the combined summary analyses. The data report was conducted in accordance with the PRISMA (ie, Preferred Reporting Items for Systematic Reviews and Meta-Analyses) Statement for Reporting Systematic Reviews and Meta-Analyses of Studies.26

Results

The pooled series deriving from the 7 studies included 1370 (epi)genotyped patients with BWS: 102 were reported to develop a total of 102 BWS-related malignant neoplasms, providing a prevalence of 7.4%. The tumor prevalence differed among the molecular subtypes: 2.5% (21/836) in ICR2-LoM, 13.8% (47/341) in UPD, 22.8% (28/123) in ICR1-GoM, and 8.6% (6/70) in patients with CDKN1C mutations (χ2P < .001). Each of the 4 subtypes had a cancer prevalence different from the other (P < .001). The pooled distribution of the histotypes described in the studies according to the molecular subtypes is summarized in Table II. The prevalence of Wilms tumor was greater in the ICR1-GoM and in the UPD subgroups (21.1% vs 6.2%, respectively, P < .001). The development of hepatoblastoma was associated with UPD (4.7%, P < .001), although observed also in patients with ICR2-LoM (0.7%) and ICR1-GoM (0.8%, P < .001). Adrenal carcinoma was exclusively observed in patients with UPD (1.5%, P < .001), and neuroblastic tumors were associated with CDKN1C mutations (4.3%, P = .003), although also observed in ICR2-LoM (0.5%) and cases with UPD (0.9%).

Table II. Pooled analysis of cancer occurrence and histotypes of the 4 molecular subgroups of BWS

	ICR2-LoM		UPD		ICR1-GoM		*CDKN1C* mutation		Total		
	n	% of total	n	% of total	n	% of total	n	% of total	n	% of total	*P*
Patients	836	61.0%	341	24.9%	123	9.0%	70	5.1%	1370		
Patients with tumor	21	2.5%	47	13.8%	28	22.8%	6	8.6%	102	7.4%	χ^2 $P < .001$*
Tumors	21	2.5%	47	13.8%	28	22.8%	6	8.6%	102	7.4%	χ^2 $P < .001$*
Histotypes											
Wilms tumor	1	0.1%	21	6.2%	26	21.1%		0.0%	48	3.5%	χ^2 $P < .001$†
Hepatoblastoma	6	0.7%	16	4.7%	1	0.8%		0.0%	23	1.7%	χ^2 $P < .001$†
Adrenal carcinoma	0	0.0%	5	1.5%		0.0%		0.0%	5	0.4%	$P < .001$‡
Neuroblastic tumors	4	0.5%	3	0.9%		0.0%	3	4.3%	10	0.7%	χ^2 <.001¶
Rhabdomyosarcoma	6	0.7%	1	0.3%		0.0%		0.0%	7	0.5%	n.s.
Pancreatoblastoma		0.0%	1	0.3%	1	0.8%		0.0%	2	0.1%	n.s.
Leukemia		0.0%		0.0%		0.0%	1	1.4%	1	0.1%	n.s.
Thyroid carcinoma	1	0.1%		0.0%		0.0%		0.0%	1	0.1%	n.s.
Liver sarcoma	1	0.1%		0.0%		0.0%		0.0%	1	0.1%	n.s.
Melanoma	1	0.1%		0.0%		0.0%	1	1.4%	2	0.1%	n.s.
Gonadoblastoma	1	0.1%		0.0%		0.0%		0.0%	1	0.1%	n.s.
Cardiac atrial tumor		0.0%		0.0%		0.0%	1	1.4%	1	0.1%	n.s.

*$P < .001$ for all 2 × 2 Fisher tests intergroup comparisons.
†Fisher tests $P < .001$ ICR2-GoM vs UPD or ICR1-GoM, and UPD vs ICR1-GoM.
‡Fisher tests ICR2-LoM vs UPD $P < .0001$, ICR2-LoM vs ICR1-GoM, P = not significant (n.s.), ICR2-LoM vs *CDKN1C*, P = not significant (n.s.).
§Fisher tests UPD vs other cases.
¶$P = .003$ *CDKN1C* vs other cases.

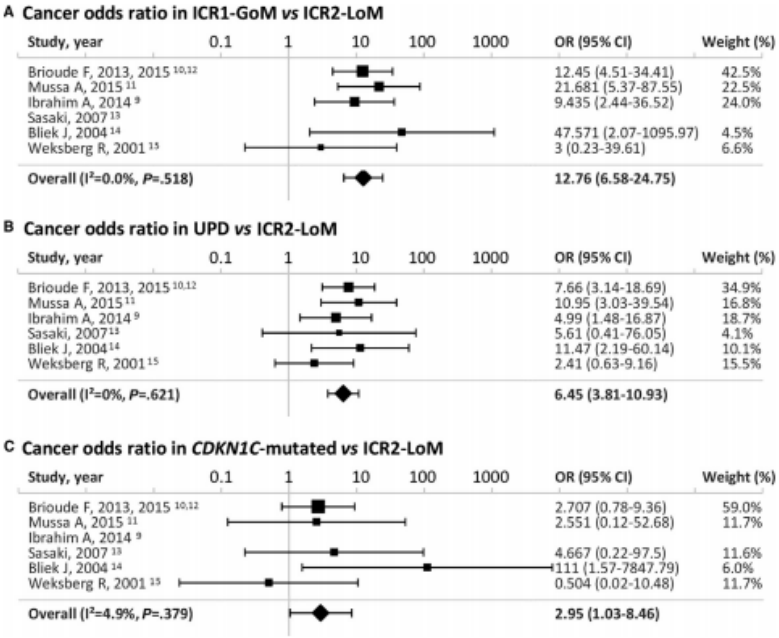
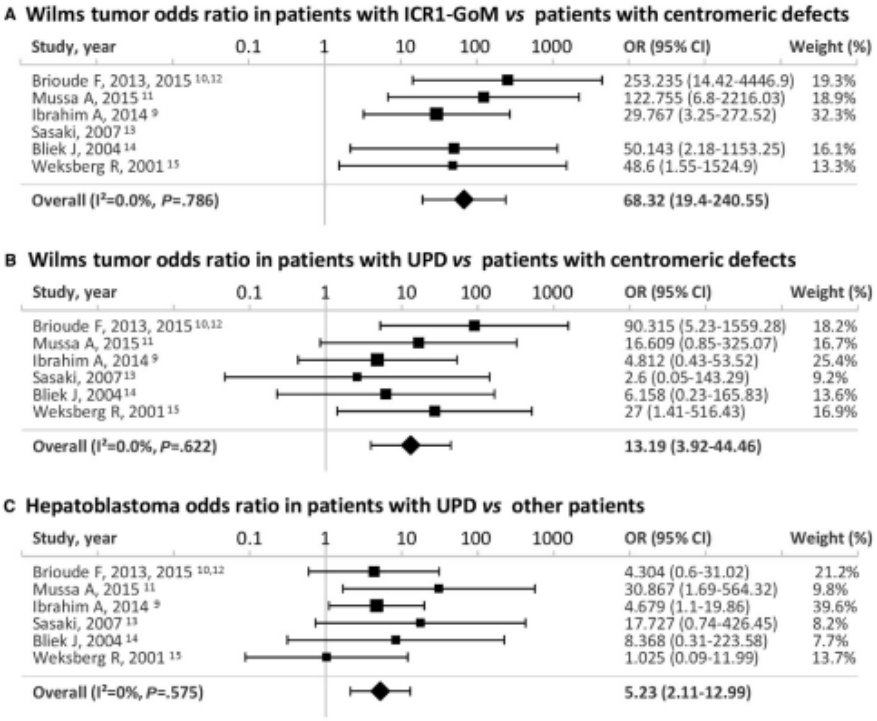


Figure 2. Meta-analysis of tumor development OR in relation to the molecular subgroup in patients with BWS. ORs and corresponding 95% CIs are shown for the comparison of tumor odds of each molecular subgroup (**A**, ICR1-GoM, **B**, UPD, and **C**, *CDKN1C* mutation) compared with patients with ICR2-LoM, based on a random-effect model.

All the significant associations found in the preliminary analysis were confirmed by meta-analysis. This revealed that, compared with ICR2-LoM, all-cancer ORs were significantly greater in the other subgroups displaying a gradient: OR 12.8 (95% CI 6.6-24.8, P < .001) in patients with ICR1-GoM, 6.5 (95% CI 3.8-10.9, P < .001) in patients

with UPD, and 2.9 (95% CI 1.03-8.5, P = .002) in CDKN1C mutated ones (Figure 2). Wilms tumor was more common in telomeric than in centromeric defects: both cases of ICR1-GoM and UPD showed ORs significantly increased as compared with centromeric defects (OR 68.3 with 95% CI 19.4-240.6, P < .001 and OR 13.2 with 95% CI 3.9-44.5, P < .001, respectively; Figures 3, A and B), and ICR1-GoM showed significantly greater OR as compared with UPD (OR 4.4 with 95% CI 2.2-8.6, P < .001; data not shown). Cases of UPD also showed an increased risk of hepatoblastoma (OR 5.2, 95% CI 2.1-13.0, P < .001) and of adrenal carcinoma (OR 7.0, 95% CI 1.7-28.9, P < .001), compared with other molecular subgroups (Figures 3, C and D). Finally, in patients with CDKN1C mutations, the ORs of developing neuroblastic tumors were significantly greater compared with the other subtypes (OR 7.2, 95% CI 2.0-26.3, P < .001; Figure 3, E).



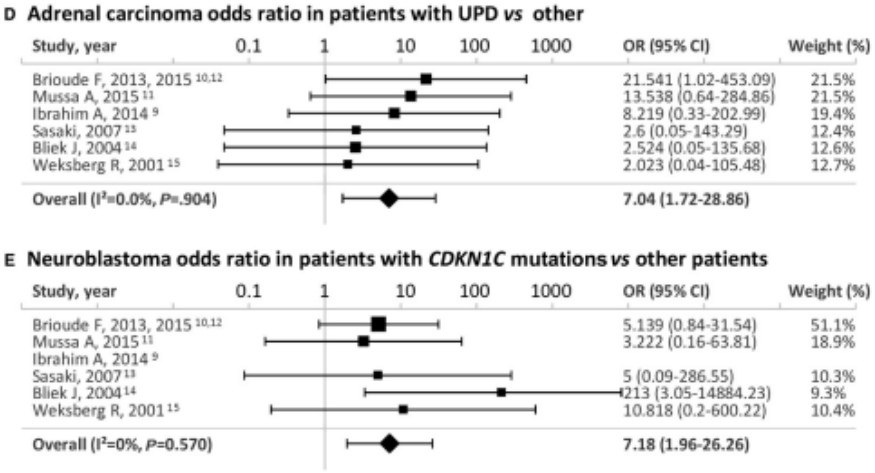


Figure 3. Meta-analysis of histotype-specific tumor development OR in relation to the molecular subgroup in patients with BWS. ORs and corresponding 95% CIs are presented for individual studies and for the overall analyses based on a random-effect model. Centromeric defects include ICR2-LoM and *CDKN1C* mutation. **A** and **B**, OR of developing Wilms tumor in patients with ICR1-GoM and UPD, respectively, compared with patients with centromeric defects; **C**, OR of developing hepatoblastoma in patients with UPD compared to those with other molecular defects; **D**, OR of developing adrenal carcinoma in patients with UPD compared with patients with other molecular defects; and **E**, OR of developing neuroblastoma in patients with *CDKN1C* mutations compared with patients with other molecular defects.

Discussion

We consolidated and further corroborated the existence of specific (epi)genotype based oncogenic risk in BWS molecular subtypes. This analysis provided a cancer prevalence roughly consistent with that observed in most previous reports—7.4%—and confirmed that the most common histotypes associated with BWS are Wilms tumor, hepatoblastoma, neuroblastic tumors, adrenal carcinoma, and rhabdomyosarcoma [Shuman et al. 2000, Rump et al. 2005, Lapunzina 2005]. Pooled data on tumor development of the 1370 patients with BWS included in this analysis robustly defined the cancer rate gradient across the 4 molecular subtypes. Moreover, the meta-analytic approach allowed us to reliably estimate the tumor development ORs of (epi)genotypes at increased risk.

We provide data on cancer histotype heterogeneity across the subtypes. Wilms tumor represents the most common histotype in patients with BWS [Rump et al. 2005]. It represents the histotype largely prevalent in the ICR1-GoM subgroup, accounting for 95% of the malignancies. The rate of Wilms tumor development in cases of ICR1-GoM is significantly greater than that observed in cases of UPD. Notably, only 1 Wilms tumor in more than 900 patients with centromeric domain defects was reported to date. This finding has implications for screening in this subgroup, which accounts for more

than 50% of overall cases with BWS. Concerning hepatoblastoma, the meta-analysis confirms our previous observation [Mussa et al. 2015]: this histotype is associated with UPD, accounting for more than one-quarter of the neoplasms in this molecular subgroup and occurring in nearly 5% of patients with UPD.

On the contrary, hepatoblastoma is very rare in the other molecular subgroups (overall 0.6%). A previously unreported finding is the association of UPD with adrenal carcinoma, an embryonal tumor poorly studied in BWS and surprisingly affecting 1.4% of patients with UPD and representing more than 10% of the tumors recorded in this group. Moreover, the novel association between CDKN1C mutations and neuroblastic tumor has been observed: this histotype represents one-half of the neoplasms associated with CDKN1C mutations and display a 4.3% incidence in this molecular subtype, much greater than that observed in the others. The rarer histotypes observed revealed no significant association with a specific molecular alteration. It is important to underline, however, that the absence of a statistically significant association might be due to the small number of the neoplasms observed. Actually, some associations appear clinically relevant although not statistically significant: 6 of 7 rhabdomyosarcomas were observed in the ICR2-LoM subgroup.

Currently, cancer-surveillance programs for patients with BWS are based on a 3-6 months' abdominal ultrasound scanning up to 8 years of age plus a 2-3 months' serum αFP determinations up to 4 years of age [Rump et al 2005] or simply by abdominal ultrasound scanning [Brioude et al 2013] and generally are applied to all patients, irrespective of molecular diagnosis and (epi)genotype. Screening procedures currently used in BWS may be rather differentiated based on the (epi)genotype, as suggested by some authors [Brioude et al 2013], and modified in the light of the results of this meta-analysis to target specific histotypes.

Concerning Wilms tumor, our analysis confirms that patients with telomeric defects likely benefit from renal ultrasound screening because cost-effectiveness in screening Wilms tumor is well proven as well as close screening intervals (3-month schedule) [Mc Neil et al 2001, Scott et al. 2006, Porteus et al. 2000]. Conversely, the measurement of the serum tumor marker αFP as a screening method for the early diagnosis of hepatoblastoma is debated, given the complexity of its interpretation in childhood [Ibrahim et al. 2014, Mussa et al. 2011, Mussa et al. 2014, MN Neil et al. 2002] and the

overall low incidence of hepatoblastoma in BWS. The variability in the natural decrease of the normal serum αFP levels during early infancy and its wide fluctuations dependent on premature birth account for further difficulties in the interpretation of αFP measurements. Moreover, frequent blood draws in children commonly is responsible for lack of adherence to screening [Zarate et al. 2009]. Data show, however, that hepatoblastoma represents the second most common embryonal tumor associated with the syndrome: BWS is the major risk factor for hepatoblastoma, accounting for an estimated risk 2280 times greater than that observed in the general population [DeBaun et al. 1998]. In our opinion, serum αFP screening is worthwhile at least in patients with UPD, given their high risk of hepatoblastoma, roughly approaching 5%.

In parallel, cancer-screening procedures for Wilms tumor are advised in several cancer predisposition syndromes with at least an estimated tumor risk around 5%,29 a rate approaching that of hepatoblastoma in patients with UPD. A greater survival rate and lower staging at diagnosis characterize cases diagnosed through specific repeated serum αFP measurement in the first 5 years of life [Trobaugh-Lotrario et al. 2014, Clericuzio et al. 2003], age after which this tumor becomes anecdotal. Remarkably, 41 of 56 hepatoblastomas in cases with BWS recently reviewed [Trobaugh-Lotrario et al. 2014] lack any details concerning the putative cancer screening procedures used. All the 14 reported cases diagnosed by screening display clearly elevated αFP levels at diagnosis in respect of reliable reference values [Mussa et al. 2011. This finding confirms the observations [Clericuzio et al. 2003] that abdominal ultrasound scanning may not be sufficient for early hepatoblastoma diagnosis and that screening patients with BWS by serum αFP allow a shift to lower stages of the diagnosis of this tumor, implying an increase in patients' survival rates. Collaborative studies are needed to verify proficiency and cost-effectiveness of serum αFP screening in this setting; of note, clinical research alternative, more cost-effective, and less-invasive methods for screening hepatoblastoma, such as αFP measurement on dried blood spot, appear promising [Mussa et al. 2014].

On the basis of the results of the meta-analysis, the use of ultrasound scanning and tumor markers screening in cases with ICR2-LoM appears questionable, given the low tumor risk and the occurrence of histotypes for which the advantage of these screening methods is unproven. Actually, in this molecular subgroup, which represents more than 50% of cases with BWS, clinical research should be focused in assessing the cost-

effectiveness of cancer-screening procedures. Moreover, future studies should assess the feasibility of the early detection of the ICR2-LoM–related malignancies and the actual impact of the detection timing on their management and treatment. Furthermore, the variegate spectrum of ICR2-LoM–related malignancies hamper the application of a single-test strategy for screening embryonal tumor in this molecular subgroup. A clinical follow-up with ad-hoc instrumental/laboratory investigations may prove to be a reasonable alternative.

The findings of UPD and adrenal carcinoma may suggest the possibility of focusing on a histotype-specific tumor screening in this molecular subgroup. Despite its low incidence in the cases with UPD, the application of a screening procedure specific for this tumor histotype may be justified by the high mortality of this aggressive cancer and the proven advantages in its diagnosis at an early stage. With this view, it is worth mentioning the adrenal carcinoma surveillance protocol undertaken in Li-Fraumeni syndrome [Custodio et al. 2013]. The authors observed that screened adrenocortical tumors were smaller and in an earlier disease stage with respect to nonscreened ones, consistent with improved survival. This protocol might also be used to screen adrenal carcinoma in patients with BWS with UPD and dehydroepiandrosterone sulfate may be measured consistent with the blood drawn for serum αFP determination up to 5 years of age.

Finally, a prevalence of 5% of neuroblastic tumors in the patients with CDKN1C mutations may justify an attempt of specific tumor screening in this subtype. A simple urine test for determination of the urinary tumor markers vanillylmandelic and homovanillic acid and/or catecholamines could be used to detect asymptomatic neuroblastomas; however, neuroblastoma screening experience in the nineties in large-scale pediatric setting [Yamamoto et al. 1995, Bessho et al. 1991, Kramer et al. 2000] led only to the detection of large numbers of indolent self-limiting neuroblastomas (IVs stage), with low impact on the related mortality rates. Therefore, further studies should assess the ability of urinary tumor markers vanillylmandelic and homovanillic acid and/or catecholamines to actually diagnose BWS-related neuroblastomas and provide advantages in terms of treatment and survival. Table III summarizes the (epi)genotype-based and histotype-targeted tumor-screening protocol we propose and implemented in our clinics: however, it should be emphasized that a general consensus agreement on tumor surveillance in BWS is strongly needed.

Table III. Proposed (epi)genotype-specific and histotype-targeted tumor surveillance protocol for patients with BWS

(Epi)genotype (% of BWS)	Target histotype	Procedures	Timing
ICR2-LoM (>50%)	Tumor incidence very low (2.5%) and extremely variegate histotype spectrum	Clinical assessment to eventually request test on specific suspicion.	4-6 mo from diagnosis to adolescence
ICR1-GoM (5%-10%)	Wilms tumor (22.8%)	Renal ultrasound scan*	3 mo from diagnosis to 8-10 y of age*
UPD (20%)	Wilms tumor (6.2%)	Renal ultrasound scan*	3 mo from diagnosis to 8-10 y of age*
	Hepatoblastoma (4.7%)	Liver ultrasound scan, serum αFP concentration†	3 mo from diagnosis to 5 y of age†
	Adrenal carcinoma (1.5%)	Clinical assessment, adrenal ultrasound, determination of serum DHEAS concentration‡	3 mo from diagnosis to age to be determined DHEAS measurement until 5 y of age paralleling hepatoblastoma tumor marker screening‡
CDKN1C mutation (5%)	Neuroblastoma (4.3%)	Urinary vanillylmandelic and homovanillic acid/urinary‡ catecholamines Abdominal ultrasound	4-6 mo from diagnosis to age to be determined‡

DHEAS, dehydroepiandrosterone sulfate.
The kind and timing of tests for early tumor recognition should be tailored according to (epi)genotype-specific tumor risk and histotype predisposition in the 4 molecular subtypes of BWS: ICR2-LoM, UPD, ICR1-GoM, and *CDKN1C* mutation.
Summary of the evidences on cancer screening protocol proposed:
*Renal ultrasound is demonstrated to allow a downgrade shift of Wilms tumor stage at the diagnosis[30] and allow more preservative treatment[33] in patients with BWS. A consensus established the validity of renal ultrasound in every genetic condition with Wilms tumor predisposition and risk greater than 5%.[29]
†Limited evidence suggests that αFP serum screening in allows a downgrade shift of the hepatoblastoma stage at the diagnosis[36] with sensitivity superior to abdominal ultrasound.
‡The use of these tumor markers as screening tools should be regarded as a proposal from the authors based on observation of other clinical/genetic conditions with increased risk of these tumors,[37] whereas data in the BWS context are completely lacking.

A limitation of this study is the lack of data concerning age at follow-up and tumor development, which was retrieved only in 2 of the studies included. For this reason, the tumor prevalence provided represents a minimum estimate. Moreover, it is not possible to design survival curves to provide directions on cancer surveillance timing and duration: the latter are therefore based on epidemiologic cancer data and empirical observations. Another drawback consists in the potential partial overlap of the studies analyzed, which cannot be completely excluded, although we believe it may only marginally affect our results. Finally, data on patients with BWS with negative molecular test have not been included and clearly deserve a specific study. Literature data on BWS with negative molecular tests are limited and show an intermediate cancer risk with multiple histotypes, in part resembling that of patients with UPD: screening by ultrasonography scanning is likely needed in this subgroup. So far the studies in which authors evaluated this issue included small cohorts and show excessively heterogeneous methodologies to draw any firm conclusion. Classification criteria to define these patients as "negative BWS" and differentiate them from other overgrowth disorders are needed first. With this respect, improvement of molecular genetics techniques will likely lead to better classify these disorders [Russo et al. 2016]. Given the retrospective nature of all the studies included, it is not possible to provide data on tumor development in 2 recently discovered entities: multilocus imprinting disorders (MLID)

and genome-wide paternal UPD. Likely, some of the patients diagnosed with as ICR2-LoM or UPD included in the meta-analysis are indeed MLID or patients with genome-wide UPD, respectively. These two novel molecular defects subtypes deserve further studies: currently, data on tumor predisposition in MLID are absent despite the fact they are estimated to represent approximately 12% of BWS [Azzi et al. 2009], and some observations in cases of genome-wide UPD suggest a relevant increase in their specific cancer risk [Kalish et al. 2013, Bertoin et al. 2015]

Chapter 4. Defining the correlation between assisted reproductive techniques (ART) and Beckwith-Wiedemann

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BACKGROUND

Since the first live birth resulting from in vitro fertilization (IVF) in 1978, a growing number of pregnancies and births derived from assisted reproductive techniques (ART) have been observed, with an astonishing growing trend over the last 15 years. According to the annual reports of the European Society of Human Reproduction and Embryology, the percentage of children born through ART in Italy increased from 1% in 2006 to 1.7% in 2010 [de Mouzon et al. 2010 de Mouzon et al. 2012, Ferraretti et al. 2012, Ferraretti et al. 2013, Kupkpa et al. 2010]. In many countries, registries for monitoring and evaluating ART pregnancies have been instituted [Dhont et al. 1999, Talaulikar et al. 2012, Sutcliffe et al. 2007, Jackson et al. 2004, Hansen et al. 2013]. Since the late 1980s, literature on outcomes has emerged [Reefhuis et al. 2009, Hart et al. 2013, Williams et al. 2014]. ART entails a greater incidence of multiple pregnancies, preterm birth and low birth weight, a significant increase in congenital defects [Reefhuis et al. 2009, Hart et al. 2013, Williams et al. 2014], and potential long-term effects including metabolic, oncologic, and psychiatric disorders. In the last decade, reports have also described an increased risk of imprinting disorders (IDs) such as Beckwith-Wiedemann syndrome (BWS), Silver-Russel syndrome, and Angelmann syndrome [Tee et al 2013, Vermeiden et al. 2013, DeBaun et al 2003, Gicquel et al 2003, Maher et al. 2003, Halliday et al. 2004].

The first report of a patient with BWS conceived through ART dates back to 1995 [Sutcliffe et al. 1995]. Three years later, Young et al [1998] described in cattle and sheep the large offspring syndrome, etiologically correlated with IVF. Interestingly, this condition is a model for the BWS phenotype [Chen et al. 2013] and its molecular mechanisms [Young et L. 2001]. In 2003, DeBaun et al. estimated a 4.6% prevalence of BWS-ART patients in their BWS cohort, 6 times higher than in the general population. Subsequently, several studies have explored this association and further investigated the phenomenon [Tee et al2013, Gicquel et al. 2003, Maher et al. 2003, Halliday et al. 2004, Chang et al, 2005, Rossignol et al. 2006, Sutcliffe et al. 2006, Doornbos et al. 2007, Lim et al. 2009, Hiura et al. 2012] with the most recent literature[Vermeiden et al. 2013] generally supporting the hypothesis that BWS is overrepresented in ART conceptions. In this study, we investigated the ART-associated risk of developing BWS

in a cohort of patients born in Piemonte, a region of northwest Italy for which data from the general population and from the registry of ART are available.

Methods

All cases were clinically reviewed at the Pediatric Genetics Unit of the Department of Pediatrics at the University of Torino. BWS diagnosis was assessed clinically and each patient was included in the cohort if they displayed at least 2 of the following BWS criteria: abdominal wall defect, macroglossia, macrosomia, embryonal tumor, ear malformations, organ enlargement, nevus flammeus, hemihyperplasia, nephrourological malformations, hypoglycemia, or family history of BWS. Macrosomia was defined as birth weight > 90th percentile according to gestational age. Piemonte, a region of northwest Italy with a population of 4 424 467 at the end of 2014, has been serviced since 1997 by a single network of pediatric genetic services. Patients were selected through a search in the regional section of the Regional Registry for Rare Diseases, which is highly complete for Piemonte. The patient search was further validated and crossmatched with (1) the archive of the Department of Pediatrics at the University of Torino, (2) the Italian Association of Patients with BWS (www.aibws.org), (3) patients' archives of extraregion pediatric genetic centers, (4) archives of the 2 laboratories offering BWS molecular analyses in Italy (Molecular Genetics Laboratory of the Instituto Auxilogico Italiano of Milan, and the Department of Environmental Sciences at the University of Naples Frederico II). Patients included were born in Piemonte between 1997 (the year in which the systematic collection of cases in the region began) and 2014.

Specific molecular tests were offered to all patients. Patients or the parents provided written informed consent. DNA was extracted from peripheral blood lymphocytes. Methylation analysis of the 11p15.5 chromosomal regions containing IC1 and IC2 was conducted in all patients and performed either by Southern blotting, CoBRA (Combined Bisulfite Restriction Analysis), or methylation-sensitive multiple ligation probe amplification (MRC-Holland, Amsterdam, Netherlands). The results obtained by these techniques have been shown to be comparable [Priolo et al. 2008, Scott et al. 2008]. In patients with suspected pUPD, a high-resolution single-nucleotide polymorphism array analysis was performed by using the CytoScan HD array (Affymetrix, Santa Clara, CA) as previously described [Palumbo et al 2014], and confirmation was obtained by

microsatellite analysis of probands and parents. CDKN1C gene sequencing was conducted in 12 patients who were selected on the basis of negativity of methylation-sensitive tests, the presence of 2 of the abovementioned BWS diagnostic criteria, and either indications of BWS or signs or malformations highly specific for CDKN1C variants (such as palatoschisis or omphalocele) [Eggerman et al. 2016].

BWS-ART patients were ascertained through the records of the Department of Health Sciences and Pediatrics at the University of Torino. The families were requested to provide ART-related clinical records and informed consent to participate in the study. BWS-ART children conceived with any ART were included in the study, such as ovarian overstimulation; IVF or embryo transfer (ET); intracytoplasmic sperm injection; intrauterine insemination; or any artificial techniques of collecting semen, such as testicular sperm extraction or testicular sperm aspiration. The variables evaluated included (1) cause of infertility, (2) drug therapy for ovarian stimulation, (3) method of collection of the semen, (4) ART method, (5) number of embryos conceived, (6) details concerning culture media, (7) embryo cryopreservation, (8) number and timing of ET and embryo implantation, (9) drug therapy postimplantation, (10) and pregnancy data.

Demographic data were gathered through the Italian National Institute for Statistics (www.istat.it), which is the main supplier of official statistical information in Italy. Epidemiologic ART data were gathered through the search in the National Registry of Medically Assisted Procreation (http://www.iss.it/rpma). This registry was established by Ministerial Decree in 2005 (Gazzetta Ufficiale 282, December third, 2005 article 11, Law 40/2004). The registry management is entrusted to a unit of the Istituto Superiore di Sanità (the National Centre for Epidemiology, Surveillance and Health Promotion) and is funded by the Italian Ministry of Health. Data collection at a national level enables immediate census results of all the centers operating in Italy and analysis of their activities and services. The aim of the registry is to assess and monitor effectiveness and safety of ART in Italy. The registry anonymously collects ART data from all the centers, the number of cycles conducted, treatment protocols used, complications, and follow-up of pregnancy and births. Data collection is performed through a Web site in which researchers at the centers enter data in anonymous aggregate form. In this study, the data entered from centers operating in Piemonte were used, starting from assisted medical procreation registry inception to the latest available consolidated data set (time range 2005–2014). Data on patients with BWS were

obtained from the regional section of the Italian Registry for Rare Diseases, as previously described [Mussa et al. 2013].

Data were compared by 2 × 2 Fisher's exact tests or, in case of expected frequencies ≥5, $\chi 2$ test with Yates correction, as appropriate. Two-tailed P values <.05 were considered significant. Data were analyzed by SPSS 15.0 (IBM Software, Armonk, NY) and Prism GraphPad 5.0 (GraphPad Software, La Jolla, CA).

Results

Data from the Italian National Registry of Medically Assisted Procreation from the 2005–2014 time range are available. In this period, live births in Piemonte were 379 872 and include 7884 newborns conceived by ART. Thirty-eight patients with BWS were born, 7 through ART and 31 naturally conceived. The percentage of live births by ART across the 10-year observational period increased considerably from the lowest value (n = 441) in 2005 (1.2% of total live births) to the highest (n = 1022, 2.8%) in 2013 (r2 = 0.707, P = .002). The concomitant (2005–2014) overall birth prevalence of BWS was 1:9996 live births, confirming previous epidemiologic results. The BWS birth prevalence in the ART subgroup was significantly higher than in the naturally conceived one (1:1126 vs 1:12 254, P < .001). Hence, the absolute per 1 000 000 live birth BWS risk in the ART group was 887.9 vs 83.3 observed in the naturally conceived group, providing a relative risk of 10.7 (95% confidence interval 4.7–24.2) with a risk attributable to ART of 804.5 per 1 000 000 live births. We summarize these data in Table 1.

TABLE 1 Epidemiology of BWS in Patients Conceived Naturally and Through ART in the Time Period 2005–2014

Conception	BWS	Non-BWS	Total Live Births	Risk (Per 1 000 000 Live Births)	Prevalence
ART	7	7877	7884	887.9	1:1126
Natural	31	371 957	371 988	83.3	1:12 000
Total	38	379 834	379 872	100.1	1:9997

In the 1997–2014 time range, 67 patients were diagnosed with BWS in Piemonte out of 663 834 newborns, providing a prevalence of 1:9908 live births. Molecular testing of the 11p15.5 region was performed on 62 patients, with the remaining 5 refusing the analysis. Twenty-one patients (33.9%) had IC2-LoM, 13 (20.9%) pUPD, 7 (11.3%)

IC1-GoM, 2 (3.3%) a CDKN1C mutation, and 19 (30.6%) had negative results. Nine out of the 67 patients with BWS (13.4%) who were diagnosed with BWS in the 1997–2014 time range were conceived through ART (Table 2). In all cases, semen was collected by noninvasive methods. None of the gametes and/or embryos were of heterologous origin, and all 9 patients were born from singleton pregnancies, even in the cases characterized by multiple ETs. In no case could chemical details of embryo culture medium be retrieved. Eight BWS-ART patients were molecularly tested: 4 had a IC2-LoM, 2 had pUPD, and 2 had negative results. In one of the patients with negative tests, both DNA extracted from blood leukocytes and from exfoliated buccal mucosa cells were analyzed, with consistent negative results. A comparison between clinical features and molecular lesions detected in the BWS-ART and BWS naturally conceived groups (Table 3) revealed a comparable phenotype, with a significant difference in the incidence of macroglossia, which was less common in the BWS-ART group (44.4% vs 80.6%).

TABLE 2 Clinical, Molecular, and ART Details of the 9 Cases of ART-BWS (1997–2014)

Patient (Birth Year)	Clinical Features	ART Method, Cause of Infertility	Fertility Drugs	Embryo and ET	(Epi)genotype
#1 (2002)	Macrosomia, macroglossia, hemihyperplasia, organomegaly, nevus flammeus	IVF, undetermined	Triptorelin acetate, recombinant human FSH, chorionic gonadotropin	1 ET day 5	IC2-LoM
#2 (2004)	Macrosomia, umbilical hernia, hemihyperplasia, kidney anomalies, organomegaly, nevus flammeus	ICSI, undetermined	—	Not reported	Genotyping refused by parents
#3 (2010)	Postnatal overgrowth, hemihyperplasia, ear creases and pits	ICSI – IVF, undetermined	Buserelin acetate, recombinant human FSH	Cryopreserved 2 y transfer day 3	pUPD
#4 (2011)	Hemihyperplasia, umbilical hernia, monozygotic twinning (healthy twin with negative molecular tests)	ICSI, undetermined	Cetrorelix acetate, menopausal and recombinant human chorionic gonadotropin	3 ET day 3	Negative
#5 (2011)	Macroglossia, umbilical hernia, nevus flammeus, vast hemangioma of the skull, hemihyperplasia	ICSI, azoospermia and endometriosis	Ganirelix acetate, GnRH, recombinant human FSH, chorionic gonadotropin	2 ETs day 4	IC2-LoM
#6 (2012)	Severe macrosomia, hemihyperplasia, macroglossia, large umbilical hernia, neonatal hypoglycemia, organomegaly	IVF – ICSI, undetermined	Triptorelin acetate, recombinant human FSH, chorionic gonadotropin	2 ETs day 3	pUDP
#7 (2013)	Macrosomia, hypoglycemia, nuchal hemangioma, nevus flammeus	IVF – ICSI, azoospermia	Triptorelin acetate, recombinant human FSH, chorionic gonadotropin	1 ET day 5	IC2-LoM
#8 (2013)	Macrosomia, nevus flammeus, hemihyperplasia, umbilica hernia	IUI, female infertility	Buserelin acetate, recombinant human FSH, chorionic gonadotropin	—	IC2-LoM
#9 (2014)	Hemihyperplasia, diastasis recti	ICSI	recombinant FSH (derived from Chinese hamster ovary)	Not reported	Negative

FSH, follicle-stimulating hormone; GnRH, Gonadotropin-releasing hormone; ICSI, intracytoplasmic sperm injection; IUI, intrauterine insemination; —, no data available.

TABLE 3 Comparison of the Clinical Characteristics and Molecular Lesions of the BWS Patients Conceived Through ART and Naturally

Clinical Features	Frequency in ART-Conceived BWS	Frequency in Naturally Conceived BWS	*P*
Macrosomia	5/9 (55.6%)	49/58 (84.5%)	.06
Macroglossia	4/9 (44.4%)	50/58 (86.2%)	.01
Omphalocele	1/9 (11.1%)	7/58 (12.1%)	.99
Umbilical hernia	2/9 (22.2%)	11/58 (19.0%)	.97
Diastasis recti	2/9 (22.2%)	16/58 (27.6%)	.97
Hemihyperplasia	7/9 (77.8%)	37/58 (63.8%)	.71
Nevus flammeus	4/9 (44.4%)	26/58 (44.8%)	.99
Hypoglycemia	3/9 (33.3%)	14/58 (24.1%)	.68
Ear malformations	2/9 (22.2%)	18/58 (31.0%)	.71
Organomegaly	3/9 (33.3%)	24/58 (41.4%)	.98
Embryonal tumors	0/9 (0%)	7/58 (12.1%)	.58
Gestational age at birth	38.1 ± 2.3 wk	37.6 ± 3.6	.67
Premature birth	2/9	11/62	.99
(Epi)genotype			
IC2-LoM	4/8	18/54	.44
IC1-GoM	0/8	7/54	.58
pUPD	2/8	11/54	.67
CDKN1C mutation	0/8	2/54	.99
Negative	2/8	17/54	.98

Discussion

Several reports confirmed the association between ART and BWS by different methodological approaches [DeBaun et al. 2003, Gicquel et al. 2003, Maher et al. 2003, Halliday et al. 2004, Chang et al. 2005, Rossignol et al. 2006, Sutcliffe et al. 2006, de Waal et al. 2015, Bodwin et al. 2007, Lidegaard et al. 2005, Kallen et al. 2005, Gomes et al. 2009] (Table 4), with relevant exceptions [Doornbos et al. 2007, Lidegaard et al. 2005]. As reviewed by Vermeiden and Bernardus [2013], by pooling the risks observed in 8 epidemiologic studies, the fraction of BWS in the ART-conceived population was significantly higher than in the general population, with a relative risk of 5.2 (95% confidence interval 1.6–7.4).

TABLE 4 Summary of the Studies Correlating BWS and ART

	ART-BWS or BWS	RR or OR (95% CI)	Prevalence	ART-BWS Cases With Molecular Test	Molecular Defects Found in ART-BWS	Kind of ART Employed	Comments
DeBaun et al[17]	7 (3/65 in BWS registry, 4.6%)	6.0 (2.0–18.2)[a]	—	6	4 IC2-LoM, 1 both IC2-LoM and IC1-GoM with excluded pUPD, 1 negative	IVF, ICSI, TESE, ovodonation	Retrospective case series comparing ART prevalence in BWS with ART rate in the general population and features of ART and non-ART BWS patients.
Maher et al[19]	6/149 (4.0%)	OR 3.5 (1.5–8.8)[a]	—	2	2 IC2-LoM	IVF, ICSI	Observed frequency of ART in BWS cohort greater than that expected on the basis of the ART rate in the general population.
Bowdin et al[44]	—	—	1:1524	1	1 IC2-LoM	IVF, ICSI	Survey of 1524 children born after ART in Ireland and central England, 1 BWS found.
Gicquel et al[18]	6/149 (4.0%)	RR 3.2 (1.4–6.8)[a]	—	6	6 IC2-LoM	IVF, ICSI	Retrospective case series comparing ART prevalence in BWS with ART rate in the general population.
Rossignol et al[32]	11/40 (27.5%)	—	—	11[b]	11 IC2-LoM	IVF, ICSI	Compares methylation status at other loci of ART and non-ART BWS patients with IC2-LoM.
Halliday et al[20]	4/37 (10.8%)	OR 17.8 (1.8–432.9) RR 9.6 (CI 3.8–24.1)[a]	1:3723	3	3 IC2-LoM	IVF, ICSI	Case-control study, estimated prevalence of BWS in IVF population ~1:4000, 9 times greater than in the general population.
Sutcliffe et al[33]	11/79 (7.6%)	OR 3.6 (1.6–7.8)[a]	—	8	8 IC2-LoM	ICSI, IVF, ovulation induction	Questionnaire-based study of ART prevalence in BWS cohort (2.9%, 95% CI 1.4%–6.3%) versus ART rate in the general population.
Tee et al[15]	14/187 (7.5%)	—	—	14[b]	14 IC2-LoM	—	Compares methylation status at other loci in patients with IC2-LoM. 50% (7 of 14) of BWS patients with an IC2 epimutation, who were conceived ART, displayed a multiple epimutation epigenotype compared with 15% (26/173) of naturally conceived patients.
Chang et al[31]	19/341 (5.5%)	—	—	6	Already reported by DeBaun et al[17]	IVF, ICSI, IUI, ovulation induction	Retrospective case series comparing features of ART and non-ART BWS patients.
Hiura et al[36]	6/70 (8.6%)	RR 12.5 (4.9–31.8)[a]	—	15	1 IC2-LoM	ICSI	Epidemiologic survey on incidence of IDs in the general population and ART rate. BWS prevalence in Japanese population 1:287 000, ART use in BWS higher than the nationwide frequency.
Doornbos et al[34]	6/71 (8.5%)	RR 6.1 (2.5–9.4)[a]	—	4	4 IC2-LoM	IVF, ICSI	Retrospective case series comparing ART prevalence in BWS with ART rate in the general population plus a questionnaire survey in ART in families with a child with BWS: after correction for the increased fertility problems of the parents, there is no increased incidence of ART-related birth of AS, PWS, or BWS children.
Lim et al[35]	25/112[c] (22.3%)	—	—	25	24 IC2-LoM, 1 negative	IVF, ICSI	Case control study to compare clinical features and molecular results of ART and non-ART BWS.
This study	9/67 (13.4%)	RR 10.7 (4.7–24.2)	1:1126	8	4 IC2-LoM, 2 pUPD, 2 negative	IVF, ICSI, IUI	—

AS, Angelman Syndrome; CI, confidence interval; ICSI, intracytoplasmic sperm injection; IUI, intrauterine insemination; OR, odds ratio; PWS, Prader-Willi Syndrome; RR, risk ratio; TESE, Testicular Sperm Extraction; —, not applicable/not available.

[a] RR derived from Vermeiden and Bernardus,[35] in which an overall pooled RR of the 8 epidemiologic studies was 5.2 (95% CI 1.6–7.4).

[b] Only patients with IC2-LoM were included in the studies.

[c] Only IC2-LoM non-ART BWS patients were included.

In our study, we provide the highest ever observed prevalence figure of BWS among ART-conceived children, 1:1126 live births, estimating a >10-fold increased risk of BWS in the ART population compared with natural conception. The reliability and strength of this estimate is based on the fact that this is the first retrospective study taking into account a defined time window and relying on durable registries. Actually, ART data have been collected in the National Registry of Medically Assisted Procreation across a 10-year observation interval, and BWS data have been collected by a comprehensive multimodal approach on the basis of the regional section of the Italian Registry for Rare Diseases. This thorough epidemiologic method allowed for the identification of almost every patient with BWS. On the contrary, most of the previous studies provided only indirect estimates by relating the ART rate observed in BWS cohorts to that observed in the general population at the time of the investigation [DeBaun et al. 2003, Maher et al. 2003, Sutcliffe et al. 2006, Doornbos et al. 2007, Hiura et al 2012] or by self-reporting [Bowdin et al. 2007]. On the basis of 4 patients with BWS conceived through ART out of 37 observed, Halliday et al [2004] directly estimated BWS overall prevalence (1:35 580 live births) and prevalence in ART pregnancies (1:3724). However, in that setting, the prevalence of BWS itself seems to be different from that reported in other countries [Mussa et al. 2013]. Researchers conducting nationwide surveys and cohort studies looking for BWS in ART-conceived children found contrasting results; no association was found in Denmark [Lidegaard et al. 2005], the Netherlands [Doornobos et al. 2007], or Sweden [Kallen et al. 2005], whereas a BWS prevalence of 1:1524 ART live births was reported in Ireland and England [Bowdin et al. 2007]. The latter figure approximates the one we have observed, although it should be taken cautiously because it is based on the finding of a single BWS case in the whole ART cohort. It is likely that the data provided in our study are more robust because our data are based on a longer observation period and relying on 7 patients with BWS conceived through ART and born in the 2005–2014 time range with highly reliable ART epidemiologic data.

We are not able to provide epidemiologic data on other IDs for which an association with ART has been hypothesized (such as Silver-Russel syndrome, Angelmann syndrome, and Prader-Willi syndrome). This study was designed to assess BWS prevalence only, and we do not have access to the complete registry data for other IDs. Furthermore, those IDs are much rarer than BWS, complicating an accurate

epidemiologic survey in both the general population and ART children. However, in the series of patients with non-BWS IDs with whom we followed up at our institution, we have not observed any who were born through ART.

The molecular defects found in patients with BWS conceived through ART consist mostly of IC2-LoM, suggesting that either subfertility or ART procedures impair either the acquisition or the maintenance of the maternal methylation at the 11p15.5 region. [Gomes et al. 2009]. Despite the increased occurrence of IDs in ART cohorts, the question is whether ART itself is the cause of the increased BWS prevalence. Rather than ART itself, it has been proposed that the genetic background of the infertile parents may be responsible for this phenomenon [Doornbos et al. 2007, Hiura et al. 2012]: it is difficult to separate the iatrogenic effect of ART procedures from the underlying infertility causes as well as maternal age and environmental exposures. Nevertheless, researchers have demonstrated in experimental studies in animal models that embryo manipulation plays a role as a direct pathogenetic mechanism for imprinting defects [de Waal et al. 2015]. Moreover, researchers for a substantial amount of studies in ruminants (cattle, sheep, and bovine) show convincingly that ART, and especially IVF, cause significant epigenetic modifications that alter the expression of imprinted genes. The most significant effect on the phenotype is bovine large offspring syndrome, which mimics the phenotypic abnormalities of human BWS, including overgrowth, an enlarged tongue, and abdominal wall defects, as well as the associated molecular defects. Indeed, aberrant CpG methylation and loss of imprinting at the KCNQ1/KCNQ1OT1 locus has been found to be associated with large offspring syndrome [Young et al. 1998, Chen et al. 2013, Hori et al 2010]. Interestingly, Chen et al [2015] have recently found imprinting defects of multiple loci in bovines affected by ART-induced large offspring syndrome, further extending the similarities with BWS and strengthening the hypothesis that ART may directly cause imprinting disturbances. Actually, more than 95% of patients with BWS conceived through ART reported to date had IC2-LoM, either associated or not with hypomethylation at other imprinted loci. Two patients with normal methylation patterns [DeBaun et al. 2003, Lim et al. 2009] and 1 with both IC2-LoM and IC1-GoM (excluding pUPD) [DeBaun et al. 2003] were reported, to the best of our knowledge. Unexpectedly, we found IC2-LoM only in half of our patients with BWS conceived through ART, whereas the remaining 4 had pUPD (n = 2) or a normal methylation profile (n = 2). pUPD cases have been confirmed by

microsatellite and single-nucleotide polymorphism array analysis, excluding multiple methylation anomalies (ie, concomitant IC2-LoM and IC1-GoM) described elsewhere [Lennerz et al. 2010]. The finding of pUPD cases in patients with BWS conceived through ART demonstrated mosaic chromosome 11 pUPD, likely resulting from postfertilization somatic recombination. This finding is relevant because most research on ART-related IDs has focused on methylation disturbances of the oocyte or the male gamete. If confirmed, our findings of pUPD cases in ART-BWS cohorts would rather support a pathogenetic role of embryo manipulation.

Research on the relationship between ART and BWS also focused on addressing the existence of differences between ART and non-ART cases in terms of phenotype, (epi)genotype, or multiple epimutation [Tee et al. 2013, Chang et al. 2005, Rossignol et al. 2006, Lim et al. 2009]. In our study, the comparison between the features of ART and non-ART patients with BWS did not reveal substantial differences between the 2 groups; only macroglossia was significantly less common in the ART group. It is worthy to note that macroglossia is currently considered one of the most sensitive and specific traits of BWS [Ibrahim et al. 2014]; this may imply that BWS cases should be suspected in ART birth even without macroglossia. Also of interest, although prematurity has been connected with BWS and is more represented in the IC2-LoM group [Mussa et al. 2016], we detected no differences in gestational age at birth between ART and non-ART patients with BWS. As in many other studies, the IVF protocols were investigated without identifying the consistency of culture media, the procreation technique, or the underlying etiology of infertility, despite having all in common ovarian overstimulation. In our study, despite a thoughtful investigation, many details of ART procedures were irretrievable. ART procedures should be included in medical records to investigate the biological mechanisms involved in the pathogenesis of related health risks. Undoubtedly, some degree of regulation is needed to improve outcomes in the field of reproductive medicine.

Conclusions

We confirmed a significant association between ART and BWS and report the highest prevalence of BWS in ART cohorts described to date: 1 in 1126 live births. In contrast with previous reports, the (epi)genotype analysis of the ART-conceived patients with BWS in our cohort revealed that pUPD is present in a consistent fraction of the cases.

This observation needs further confirmation and research because it may imply that ART is implicated in the pathogenesis of genomic events besides methylation anomalies. Nevertheless, the significantly higher rate of ART conception among patients with BWS suggests that continued investigation into the effect of ART on human imprinting is warranted, as well as the long-term consequences of human embryonic manipulation that could play a role in the developmental origin of adult diseases. The association of IDs with ART should be mentioned in the informed consent proposed to couples considering ART-based reproductive choices: there is a clear increase in the relative risk of BWS, although the absolute risk for BWS is still small. As a final point, we highlight the need for awareness in the scientific community and in the general population of ART-associated health risks that should be taken into account in the complex cultural debate on human procreation, a major issue in modern public health politics.

Chapter 6. Beckwith-Wiedemann and adulthood

This work has been accepted as

Andrea Gazzin, Diana Carli, Fabio Sirchia, Cristina Molinatto, Simona Cardaropoli, Giuseppe Palumbo, Giuseppe Zampino, Giovanni Battista Ferrero, Alessandro Mussa. **Phenotype evolution and health issues of adults with Beckwith-Wiedemann Syndrome.** American Journal of Medical Genetics Part A

BACKGROUND

Very few is currently known on BWS spectrum (BWSp) natural history and presentation in adulthood: information on BWSp impact later in life is limited and rarely reported [Greer Kj. Et al. 2008]. However, this issue appears to be of paramount importance in clinical practice as parents and young adult patients themselves have a variety of questions about possible medical problems arising in adulthood and later consequences of childhood health issues. Adolescents and adults frequently ask about fertility, pregnancy, tumor risk and later health status. Anecdotal experiences allow physicians to provide only unsatisfactory information. With these premises and prompted by patients' associations (Italian Association of Patients with BWSp, Associazione Italiana Sindrome di Beckwith-Wiedemann, AIBWS, www.aibws.org), here we investigated these issues.

Methods

Patients

We recruited patients with BWSp aged > or = 18 years with a search conducted through the BWS Registry of the Pediatric Genetics of our Institution and information gathered through AIBWS, data were acquired after obtaining the informed consent. BWS diagnosis was assessed clinically and/or molecularly according to recent diagnostic criteria [Brioude et al. 2018].

Data collection

Data collection was conducted through 1) administration of a standard questionnaire and 2) revision of clinical documentation, including recent medical visits or, when unavailable, conducting a physical exam. Telephone interviews, e-mail communication, contacts with the general practitioners and personal examination of the available clinical documentation including pictures were used to obtain the data. Data acquisition was divided in the following sections and items: 1) BWSp diagnosis. Information about BWSp phenotype and genotype, including ages at diagnosis, molecular tests, specific procedures and timing of follow up and tumor surveillance were acquired; 2) Correction of BWSp related anomalies. Each BWSp clinical feature were investigated, with the specific medical or surgical strategy adopted, as well as the evolution of the defect, and

the impact on the overall health status at the time of the study. Information about macroglossia and associated orthodontic, swallowing and speech anomalies, the need for reductive glossectomy or orthodontic intervention were obtained. Regarding lateralized overgrowth, attention was focused on the affected body part and, in case of lower limb involvement, the orthopedical surgery or orthoses correction needed. For patients affected by abdominal wall defects information about surgery required and aesthetical revision were acquired. In-depth details were also recorded for neonatal hypoglycemia and nephrourological conditions; 3) Follow up and cancer surveillance procedures. Attention was focused on specific procedures and timing of follow up and medical checks concerning tumoral aspect of BWSp (i.e. abdominal ultrasound, alpha-fetoprotein measurement, etcetera); 4) Growth. Current stature, weight and cranial circumference were acquired. Standard Deviations (SDS) was calculated for each patient based on local standards [Cacciari et al. 2012] and definitive stature was compared with parental height if known; 5) Qualification, functioning and physical activity. Educational level, current and previous jobs, sport activities were obtained; 6) Prenatal findings, pregnancy and delivery data and psychomotor development. In this section we collected information about prenatal findings, pregnancy and delivery complications, as well as data about development milestones, learning difficulties and eventual intellectual disability 7) Adult health condition. Data about current and through-adulthood health status diseases or medical issues arose in adulthood were investigated. To each subject was primarily asked in general terms if since 18 years of age he/she had met relevant health issues requiring a significant medical intervention 8) Tumor data were collected, focusing on histology, age of diagnosis, methods of diagnosis (accidental diagnosis, related symptoms or tumoral screening) and therapeutic strategies. 9) Reproduction and procreation. We surveyed data about fertility (attempts to conceive, fertility exams and tests), pregnancy, delivery and health status of the patients' offspring.

Results

Forty-two patients were contacted and 34 (aged 18 to 58 years, mean age 28.5±9.9, 18 females and 16 males) agreed to participate in the study. Thirty patients had a clinical diagnosis with a BWSp score ≥ 4 and four patients with a clinical score of 3 points had a positive molecular test confirming the diagnosis. Molecular tests were performed in 31 subjects, at mean age of 19.1±15.0 years: 14 patients presented 11p15.5 Imprinting

Center 2 Loss of Methylation (IC2-LoM, 41.2%), 2 KCNQ1 microduplication [Chiesa et al. 2012] and 1 microdeletion [Zollino et al. 2010] associated with IC2-LoM, (8,8%), 2 Imprinting Center 1 Gain of Methylation (IC1-GoM, 5,9%), 1 microdeletion of the IC1 associated with IC1GoM (2,9%), 5 had 11p15.5 Paternal Uniparental Disomy (UPD(11)pat, 14.7%), 1 CDKN1C mutation (2.9%). Five out of the 31 patients analyzed resulted negative at the molecular tests (16.1%), while molecular analysis was not performed in 3 patients out of 34 (8.8 %). Age at diagnosis ranged from birth to 41 years (mean 5.0±9.9 years), in 17 cases (50.0%) diagnosis was formulated at birth due to easily recognizable features, in other three cases (8.8%) in the first year of life.

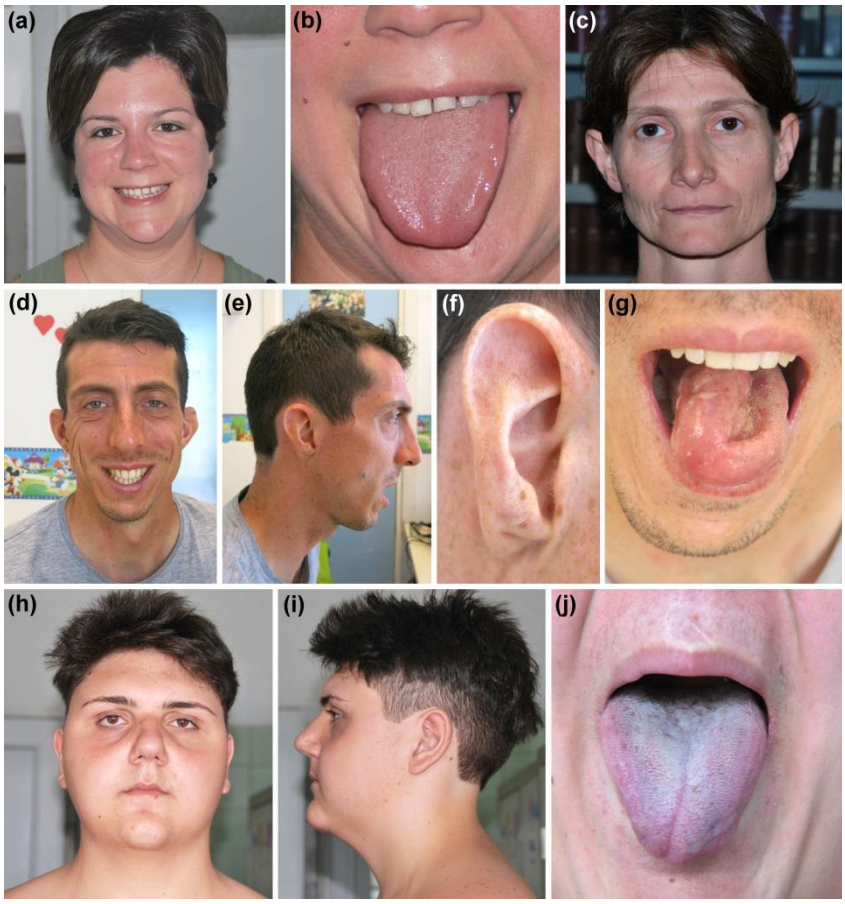
1) **BWSp features and correction of BWSp-related malformations** - Table 1 shows the clinical features and the related treatment. Figure 1 shows facial characteristics and macroglossia of adult patients. Overall, 52.9% (18/34), 14.7% (5/34) and 17.6% (6/34) patients underwent one, two or more surgical interventions, respectively, and five patients never underwent surgery. Surgical treatment was required for tongue reduction, cryptorchidism, lower limb length discrepancy correction, mandibular advancement, abdominal wall defects correction (all surgically corrected at birth in case of omphalocele), surgical removal of tumors, penis surgery due to recurvation and labia minora reduction due to asymmetry.

Table 1 ― BWSp features in the study group.

Feature	Cases/Sample †
Macroglossia	**31/32 (96.9%)**
Hemi-macroglossia	6/31 (19.4%)
Surgery	14/31 (45.2%)
Surgical tongue reduction	11/31 (35.5%)
Maxillary advancement/mandibular retraction	5/31 (16.1%)
Multiple maxillofacial surgical corrections	3/31 (9.7%)
Orthodontic and Speech anomalies	15/31 (48.4%)
Orthodontic therapy	18/29 (62.1%)
Speech therapy	9/29 (31.0%)
Birth weight > +2 SDS	13/23 (56.5%)
Final height > +2 SDS	15/34 (44.1%)
Lateralized overgrowth	**22/33 (66.7%)**
Lower limb length discrepancy	20/33 (60.6%)
Surgically corrected	6/20 (30.0%)
Treated with orthoses only	4/20 (20.0%)
Upper limb overgrowth	8/22 (36.4%)
Facial asymmetry/overgrowth	7/22 (31.8%)
Abdominal wall defect	**24/31 (77.4%)**
Omphalocele	12/24 (50.0%)
Umbilical hernia (1 surgically reduced)	7/24 (29.2%)

Inguinal hernia (1 surgically reduced)	6/24 (25.0%)
Diastasis recti	3/24 (12,5%)
Neonatal hypoglycemia	**12/31(38.7%)**
Persisting through infancy	5/12 (41.7%)
Treated with diazoxide	3/5 (60.0%)
Pancreatectomy	1/12 (8.3%)
Urinary anomalies	**12/32 (37.5%)**
Cystic kidney	6/12 (50.0%)
Renal agenesia	1/12 (8.3%)
Ureteral malformation	1/12 (8.3%)
Nephrolithiasis	5/12 (41.7%)
Recurrent urinary tract infections	2/12 (16.7%)
Cryptorchidism	**7/16 (43.8%)**
Bilateral cryptorchidism	4/7 (57.1%)
Malignant tumors	**8/34 (23.5%)**
Wilms' tumor	4/34 (11.8%)
Hepatoblastoma	1/34 (2.9%)
Early-T acute lymphoblastic leukemia	1/34 (2.9%)
Intratubular germ cell neoplasia	1/34 (2.9%)
Testicular Sertoli-cell tumor	1/34 (2.9%)
Benign tumors	**4‡/34 (11.8%)**
Mammary fibroepithelioma	2/34 (5.9%)
Non-functional adrenal adenoma	1/34 (2.9%)
Hepatic angioma	1/34 (2.9%)
Uterine myoma	1/34 (2.9%)

† Sample included cases from the whole cohort with information available. ‡ One patient with a malignant tumor had also a benign one (ID #*22*).



2) **Follow up and cancer surveillance** - Twenty-six patients (76%) underwent cancer surveillance in infancy undergoing quarterly abdominal ultrasound (up to 8 years of age) and, all but three of them, with serum alpha-fetoprotein measurement (up to 4 years of age). Three patients still undergo abdominal ultrasound for nephrourological conditions, while further five by individual initiative. Eight patients never performed cancer surveillance in childhood, 7 because of a late diagnosis, made at 8, 11, 15, 16, 23, 28, 41 years, respectively.

3) **Growth** - Final height was > +2 SDS in 15 patients (44%). Mean height SDS was +1.33 ±1.50, range from -2.32 to +3.80. In 26 subject parents' anthropometric data were available so it was possible to compare final height to the genetic target: 15 (57.7%) showed height above their genetic target.

4) **Educational level, social inclusion and physical activity** - Educational level in the cohort is quite heterogeneous: four patients achieved university degree and five are successfully performing university studies, while 19 patients obtained or are obtaining a secondary school graduation. Four subjects obtained primary school graduation. Four patients failed and had to repeat the same grade. Fourteen (66.7%) of the 21 adult patients with available information, were gainfully employed. Regular physical activity was performed by 22 subjects, seven on a competitive level, although lower limb length discrepancy was present in six of them.

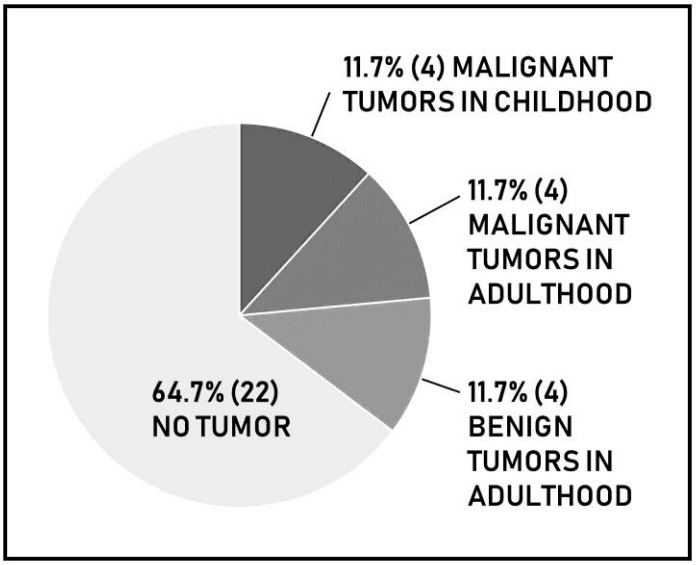
5) **Prenatal findings, pregnancy, delivery and developmental milestones** - Prenatal tests detected fetal anomalies consistent with BWSp (such as macrosomia and/or placentomegaly, increased abdominal circumference, omphalocele, elevated nuchal translucency) or elevated alphafetoprotein levels in mother's serum in 12 patients out of 22 for whom this information was available. In six cases (17.6%) spontaneous delivery was complicate by obstructed labour and in 12 cases (35.3%) caesarean section was necessary because of omphalocele, long/thick umbilical cord with loops, failing of fetal presentation during labor, breech presentation or suspected fetal hypoxia. Developmental delay or mild cognitive impairment was reported in nine (26.4%) out of 34 patients. In most cases the developmental delay was classified as mild, mostly consistent with speech delay, allowing the successfully achievement of secondary instruction, although special school was required in two cases. Neurodevelopmental outcome was more severely impaired in patient #15, probably related to the

chromosomal anomaly [Zollino et. Al. 2010] and patients #8 and #14, likely as a consequence of neonatal hypoxic ischemic encephalopathy or recurrent hyperinsulinemic hypoglycemia. Patient #8, suffered by neonatal asphyxia consistent with prolonged obstructed transvaginal delivery and macrosomia. He suffered from severe neonatal hypoglycemia and recurrent hypoglycemic episodes during infancy up to 12 years of age, requiring diazoxide treatment, complicated by drug resistant epilepsy. He was unable to work, but achieved first level secondary school education. Patient #14 suffered perinatal hypoxia consistent with delivery complication. Neurodevelopmental delay was diagnosed in the first year of age, associated to absence seizure. Speech was delayed (started at 3 years of age), autonomous walking was fully achieved at 7 years and full sphincteric control was only partially achieved. Stuttering and pronunciation deficit improved with speech therapy but was persisting at time of visit. He was able to achieve secondary school diploma with special program.

6) **Adult health condition** - Table 2 shows relevant clinical conditions besides BWSp features. Table 3 lists adult conditions which are allegedly consequent of pediatric phenotype.

7) **Neoplasm** (Figure 2) — Wilms' tumor (WT) has been diagnosed in four patients, at 2, 3, 5 and 10 years of age, respectively: in three cases WT was detected by abdominal ultrasound, either during cancer screening (n=2) or in the context of an intercurrent illness at 3 years of age, with subsequent diagnosis of BWSp (n=1). In one case WT was diagnosed at 5 years of age after the finding of hematuria. Adult onset malignancies occurred in four patients. Patient #23 developed hepatoblastoma at the age of 22 years, requiring liver transplantation, duodeno-cephalo-pancreatectomy, multiple cycles of chemotherapy and surgical removal of pulmonary metastasis. In spite of intensive treatment, he died five years after diagnosis. Patient #15 developed a T-Acute Lymphoblastic Leukemia (ALL) at 21 years of age. The girl was diagnosed with V617F JAK2 positive essential thrombocythemia at the age of 6 years. She was treated with AEIOP-BFM 2009 protocol and underwent allogenic bone marrow transplantation from HLA identical sibling, with a graft failure two months after and deceased after relapse at 23 years of age. Patient #21 developed a non-functional adrenal adenoma at the age of 22 years and testicular Sertoli-cell tumor at 24 years, successfully removed and patient #29 developed testicular Intratubular Germ Cell Neoplasia, Unclassified (IGCNU) at 27 years of age. Figure 3 shows Kaplan-Meier curve of the tumor-free probability in BWS

up to 30 years of life. Benign adulthood-onset tumors occurred in further three patients with two mammary gland fibroma, one uterine myoma and one hepatic angioma (table 4).



8) **Reproduction issues and procreation** — Four patients, two females and two males, successfully procreated, three physiologically and one through artificial reproductive techniques. Patient #9 (female, IC2-LoM) conceived a male at the age of 31 and a female at the age of 33 years, both from uncomplicated pregnancy with normal prenatal screening tests. Delivery was spontaneous for the male and by caesarean section for the female due to transverse fetal position during labor. Both children were in good health, had normal psychomotor development and no BWSp features. Patient #31 (female) and #32 (male) were siblings. In their family segregates an intragenic inverted microduplication of 160-kB within KCNQ1 exons 10 responsible for both Long QT syndrome type 1 (LQTS1) [Valente et.al. 2019] and IC2-LoM [Chiesa et al. 2012]. They physiologically conceived and had children with unrelated partners. Patient #31 (female) had a miscarriage and two daughters; the younger was born with omphalocele, macroglossia, facial nevus flammeus, post-axial hexadactyly and auricular pits consistent with BWSp. Molecular tests showed that she inherited the intragenic inverted microduplication of KCNQ1 segregating in the family, while her older sister was in good health and did not inherit the molecular anomaly: the mother was diagnosed with BWSp after the daughter. Patient #32 (male) had two children, a female at 34 years of

age and a male at 35, both born after uneventful pregnancies and showing normal psychomotor and physical development. The female was affected by absence seizure controlled with valproate and didn't carried the intragenic inverted KCNQ1 microduplication segregating in the family. Conversely, the male was diagnosed with LQTS1 by electrocardiography and by the presence of the intragenic inverted microduplication of KCNQ1: he undergoes cardiologic follow-up without any complication. Patient #18 (male) was infertile (oligozoospermia and teratozoospermia) and underwent a cycle of homologous In Vitro Fertilization (IVF) to conceive a female child. The latter was diagnosed with isolate right renal agenesis during pregnancy. The patient had no cryptorchidism: no specific cause of infertility was detected, and sperm analysis was normal.

Overall, seven of the 16 males (43.8%) were affected by cryptorchidism, bilateral in four cases. Four of them presented azoospermia or infertility. In three cases fertility tests were not performed. Patient #3 had bilateral orchidopexy at 4 years and had azoospermia. Patient #8 was affected by unilateral cryptorchidism and had his left testicle removed: no further investigation on his fertility status was performed nor he tried to conceive. Patient #14 received bilateral orchidopexy at 5 years of age and never attempted to conceive nor was tested for fertility. Patient #16 received bilateral orchidopexy at 6 years, was diagnosed with azoospermia at 19 years, consistent with left testicle atrophy and hypotrophy of the right one. Patient #20 had left cryptorchidism corrected at the age of 2 years; his subsequent fertility status was undetermined. Patient #21 underwent bilateral orchidopexy at the age of 8 years and showed azoospermia: at 24 years of age he was diagnosed with testicular Sertoli-cell tumor and underwent orchifunicolectomy. Patient #29 had left cryptorchidism surgically treated (age data was not available) and was subsequently diagnosed with azoospermia: he had testicle biopsy showing right testicle atrophy and an Intratubular Germ Cell Neoplasia, Unclassified (IGCNU) of the left gonad, which was removed.

The eight remaining male patients without cryptorchidism never attempted to conceive and none was ever tested for seminal anomalies. As concerns females, except for patient #9 and #31, none of the 16 remaining women attempted to conceive or referred gynecological or hormonal issues implicated in fertility, with the exception of mild common menstrual disorders and benign tumors showed in Table 4.

Discussion

Despite adulthood health status represents a relevant concern for patients affected by BWSp and their parents, information on these issues are barely mentioned in literature. Greer et al [2008] described a pedigree with CDKN1C mutation responsible for familial BWSp with four adults having fertility issues (azoospermia, low count and motility, abnormal morphology) and hearth anomalies, suggesting echocardiographic follow-up and semen analysis in adulthood. Other authors reported adults with BWSp and renal anomalies with recurrent urinary tract infections and kidney stones [Clouston et al. 1989], severe renal function impairment following diffuse nephroblastomatosis and Wilms tumor [Kulkarni et al. 2002] hearing deficiency as a consequence of stapedial fixation [Hopsu et al. 2003], genital anomalies [Clouston et al. 1989, Aleck 1989], partial bowel malrotation [Clouston et al. 1989], pituitary adenoma [Brioude et al. 2016], long-QT syndrome [Gurrieri al. 2013], psoriasis [Romanelli et al. 2010], hypothyroidism and thyroid adenoma [Cardarelli et al. 2010].

In this study a consistent number of adults with BWSp are described providing an initial view on the natural history of the condition. Most of medical issues in adulthood are conceivably evolution of BWSp infant features or consequences of their surgical correction. The majority of patients with BWSp undergo at least one surgical intervention. It is interesting to note that, in spite of the surgery performed (overall 50 in 29 patients), only few of them obtained full correction of the defect: indeed, such interventions were rarely judged fully satisfactory by the patients themselves. Although, surgery usually ameliorates the health status, often the features which required intervention persist in adulthood, resulting in compromised function or leading to esthetical concerns. This observation is not trivial, especially from the patients' point of view and underlines the need for specific research and improvement in this setting.

Macroglossia is well known to be the most common feature in patients affected by BWSp. Orthodontic anomalies, speech disturbances and swallow difficulties persisting in adulthood were reported by nine patients. These findings were present in both patients treated by orthodontic devices and those surgically treated, confirming that patients affected by macroglossia, even after surgical reduction, may not achieve complete normal function [Tomlinson et al. 2007], in spite of the absence of sensory losses after tongue reduction [Matsumoto et al. 2014].

Lateralized overgrowth with or without lower limb length discrepancy was the plausible cause of scoliosis and recurrent back pain described by four patients. Three of them were affected by lower limb length discrepancy (two surgically corrected, one functionally compensated by orthoses). Two of them performed competitive and two of them amateur sport activity.

Pancreatectomy for persistent hyperinsulinism was the cause of iatrogenic diabetes mellitus in one patient. Also nephrourological health issues during adulthood were reported: 70% of patients with nephrourological anomalies had frequent recurrent urolithiasis or urinary tract infections in adulthood [Mussa et al. 2014].

Obstructed labour secondary to fetal macrosomia and the possibly related neonatal hypoxic-ischemic encephalopathy are described in medical literature as a possible cause of intellectual disability in BWSp [Elliot et al. 1994, Pettenati et al. 1986]. Morbidity related this issue appears to be confirmed in our cohort: the three patients with hypoxic-ischemic encephalopathy showed a mild to severe degree of intellectual disability and seizures. However, all these cases also were affected by persistent hyperinsulinemic hypoglycemia at birth and through infancy, that could have played a role in the neurodevelopmental defect.

Thirty-one percent (5 out of 16) of the male patients in the cohort were infertile and 25% (4 out of 16) showed azoospermia in sperm analysis. Azoospermia was present in 4 adults: three had bilateral cryptorchidism and one unilateral. Overall, half of the males were affected by cryptorchidism and underwent a not timely surgical correction of cryptorchidism. As an early orchidopexy has been shown to be key for a proper testicular reproductive function [Canavese et al. 2009, Feyles et al. 2014], a late correction was a plausible explanation for the high rate of infertility encountered. This further underlines that, as in nonsyndromic children, a timely orchidopexy is of paramount importance in patients with syndromic presentation [Chan et al. 2014] and surgery should not be postponed during the first years of life although a definite diagnosis has been made or other medical interventions are required. However, the finding of azoospermia in an adult with CDKN1C mutation and unilateral cryptorchidism is relevant. Four similar cases have been described by Greer et al. [2008] in a family with the same molecular defect: three had documented severe abnormalities of spermatogenesis (only one with cryptorchidism) and the other had

infertility and testicular atrophy by clinical examination. As discussed, a mutation in CDKN1C might affect the expression of ZNF215, a zinc-finger protein located within the IC2 domain and important for a regular spermatogenesis [Gianotten et al. 2003]. Indeed, male BWSp subfertility appears a potentially relevant issue for BWSp adult patients, either as a consequence of cryptorchidism or a primary dysfunction of the testes, and further studies are therefore warranted in order to manage and preserve the reproductive function of male BWSp patients. As far as it concerns females, except for two patients, none of the remaining reported attempt to conceive, therefore we are unable to offer further information about female fertility.

The most relevant issue concerning health in adulthood is tumor predisposition. Literature is scattered of anecdotal reports on adult-onset tumors including adrenal adenoma [Hayward et al. 1988], bilateral pheochromocytoma [Bémurat et al. 2002], astrocytoma [Aleck 1989], acute myeloid leukemia [Houtenbos 2002], ACTH-secreting pituitary adenoma [Brioude et al. 2016], virilizing adrenocortical tumors [Bertoin et al. 2015, Romanelli et al. 2011], thyroid adenoma [Cardarelli et al. 2010], breast cancer [Fleisher et al. 2000] and fibroadenoma [Bertoin et al. 2015]. However, general clinical experience denies specific increase of tumor risk in adults with BWSp and it is a common convincement that it approaches that observed in the general population after the first decade of life, but no study that specifically evaluated this issue on a large cohort has been performed. In this study we have observed a tumor risk of 11.7% during childhood consistent with that reported in literature. Surprisingly, we observed in adults the same number of malignant tumors we documented in childhood. This observation doubles the overall risk previously estimated in BWSp raising tumor rate to 23.5%. Likely, this data is overestimated due to the study design. First, a collection bias is plausible, as more than half of the cohort described has been gathered though a search among the associates of AIBWS, with an over representation of adult with relevant health issues. Second, one of the testicular tumors observed is notably associated with delayed intervention for cryptorchidism. It is anyhow interesting the observation in young adulthood of three tumors usually observed in childhood (ALL, hepatoblastoma and Sertoli-cell testicular tumor), two of them leading to patients' death. Finally, it should be noted that one case of WT was diagnosed at the age of 10 years, beyond the end of the ultrasound screening recommended in infancy.

In conclusion, in this study it is described the first large cohort of adults with BWSp. Although no novel specific aspect of BWSp emerged, adult patients present several medical issues related to complications of developmental defects characterizing the pediatric phenotype. These observations underlie the preventive role of follow-up strategies in childhood and evidence the need for an improvement in treating the medical problems connected with BWSp in the first years of life. With the limitation discussed, our data show that tumor rate in BWSp cumulatively raises 23.5% including young adulthood, but the small number of patients and tumors described do not allow to provide a precise estimate of cancer risk in adulthood and mostly do not imply any revision of the proposed tumor screening protocols. However, this issue deserves undoubtedly further investigation.

Table 2 — List of health issues and medical conditions in childhood and adulthood

ID	Age (years)	Genotype	BWSp phenotype	Sex	Relevant † health issues	
					In infancy and adolescence	In adulthood
1	30	LoM IC2	MG, UH	F		
2	18	Negative	LO, MG, MS, NTH	M		Scoliosis and recurrent back pain
3	35	LoM IC2	LO, bilateral C, IH, OM, NTH, renal cysts	M		Azoospermia
4	58	Negative	MG, O, NTH	F	Recurrent severe urinary tract infections	Uterine myoma. Maculopathy, two episodes of transitory ischemic attack at 42 y and a third at 45 y resulting in left ear central hypoacusis
5	18	Negative	LO, emi-MG, O, UH, MS, OM, left kidney agenesia, right kidney malformation	F	Brain Chiari malformation (occasional finding at MRI for recurrent lipothymias)	Mammary gland fibroadenoma, scoliosis, atopic dermatitis, labia minora overgrowth with asymmetry (surgical reduction)
6	23	UPD(11)pat	LO, MG, UH, IH NPH, renal cysts	M	Perinatal Hypoxic-Ischemic Encephalopathy, hyperinsulinemic hypoglycemia recurrent through infancy until 9 y, mild intellectual disability	Drug-resistant epilepsy
7	19	GoM IC1	LO, MG, OM, WT	F		
8	45	GoM IC1	LO, MG, IH, NPH, OM, MS, left C, unilateral renal agenesis	M	Perinatal Hypoxic-Ischemic Encephalopathy, hyperinsulinemic hypoglycemia recurrent through infancy, severe intellectual disability	Drug-resistant epilepsy
9	43	LoM IC2	MG, O, NTH, MS	F	Volvulus at 50 days	
10	18	LoM IC2	LO, MG, O	M		
11	25	LoM IC2	LO, MG	F		
12	18	LoM IC2	LO, MG, MS	F	Recurrent syncopal episodes (vasovagal)	
13	20	UPD(11)pat	LO, MG, urolithiasis	F		Recurrent urinary tract infections

14	24	Not tested	LO, MG, UH, NPH, urolithiasis, C	M	Perinatal hypoxic-ischemic encephalopathy, hyperinsulinemic hypoglycemia recurrent through infancy, severe intellectual disability	Nasal polyposis, absence seizure, right ear neurosensorial deafness, psychiatric intermittent explosive disorder requiring involuntary commitment
15	-	LoM IC2, IC2 deletion [14]	MG, MS, UH, OM, NTH	F	Essential Thrombocythemia JAK2 V617F positive, mild psychomotor delay, facial dysmorphisms [14]	Early-T acute lymphoblastic leukemia
16	30	Negative	MG, UH, OM, bilateral C	M		Azoospermia
17	25	LoM IC2	LO, MG, UH, GNP, MS	F	Type 1 diabetes mellitus	
18	42	UPD(11)pat	LO, UH, WT, renal cysts	M		Infertility, genital surgery for *recurvatio penis*, recurrent urolithiasis
19	22	LoM IC2	LO, O, UH, OM, renal cysts, severe MG, glabellar nevus flammeus	M	Corpus callosum dysplasia and congenital abnormalities of the dural venous sinuses, severe macroglossia requiring intubation at birth, bronchiolitis (respiratory support, tracheostomy at 1,5 year of life), left vocal cord paralysis and dysphonia, Patent Ductus Arteriosus surgically corrected (1 y)	Scoliosis, recurrent back pain
20	18	GoM IC1, IC1 microdeletion	LO, MG, OM, ureteral malformation, WT, left ureteral malformation, left C, MS	M	Recurrent otitis (myringoplasty)	Mild mitral valve insufficiency, inflammatory bowel disease, obesity (class III), obstructive sleep apnea, primary hypertension
21	41	LoM IC2	LO, MG, MV, MS, sponge kidney, nephrocalcinosis, bilateral C, auricular pits	M		Adrenal adenoma, Sertoli-cell testicular tumor, primary hypertension, atrial fibrillation, azoospermia, recurrent urolithiasis
22	18	Negative	LO, emi-MG, O, NPH, MS	F	Brain Chiari type I malformation, total pancreatectomy for persistent intractable hyperinsulinism	Scheduled abdominal plastic surgery for multiple abdominal laparotomies, iatrogenic diabetes mellitus

23	-	LoM IC2	LO, MG, O, OM, renal cysts, MS	M		Hepatoblastoma
24	19	LoM IC2	LO, MG, O, MS	F		
25	20	LoM IC2	LO, MG, UH	F		Chronic autoimmune thyroiditis
26	29	Not tested	LO, MG	F		
27	30	LoM IC2	MG, O, urolithiasis, MS, NTH	F	Abdominal debridement due to adhesion	Aesthetic abdominal wall surgery for scars
28	41	UPD(11)pat	LO, MG, NTH, OM	F		Scoliosis and recurrent back pain
29	31	*CDKN1C* mut	MG, O, IH, left C	M		Left testicle IGCNU ‡, facial epidermal nevus removal, azoospermia, tibial varism
30	25	Not tested	LO, emi-MG, MS	F		Recurrent migraine, alopecia
31	37	microdup KCNQ1 - LoM IC2	O, MS	F		Long QT Syndrome type 1
32	38	microdup KCNQ1 - LoM IC2	MG	M		Long QT Syndrome type 1, asthma
33	19	UPD(11)pat	LO, MG, WT	M		
34	28	LoM IC2	LO, MG, O, NPH, OM, auricular pits	M	recurrent through infancy hyperinsulinemic hypoglycemia	Chronic non-specific colitis, intestinal resection due to obstruction, asthma

Table 3 — Incidence of adulthood medical issues and correlation with pediatric BWSp-related features.

BWS features		**Frequency**	**Adulthood sequelae allegedly connected**	**Frequency**
Macroglossia		31/32 (96.9%)	Persisting speech, pronunciation or swallow difficulties	9/31 (29.0%)
Lateralized overgrowth	Presence of lower limb length discrepancy	20/33 (60.6%)	Scoliosis, back pain	3/22 (13.6%)
	Absence of lower limb length discrepancy	2/33 (6.1%)		1/22 (4.5%)
Abdominal surgery		13/33 (39.4%)	Aesthetic surgery for abdominal scars	1/13 (7.7%)
Urinary anomalies †		12/32 (37.5%)	Recurrent episodes of urolithiasis	4/12 (33.3%)
			Recurrent urinary tract infections	2/12 (17.7%)
Neonatal macrosomia		13/23 (56.5%)		
	Obstructed labour	6/23 (26.1%)‡		
	Neonatal hypoxic-ischemic encephalopathy	3/13 (23.1%)	Drug-resistant epilepsy or absence seizure right ear neurosensory deafness, psychiatric intermittent explosive disorder §	3/34 (8.8%)
Neonatal hypoglycemia		12/31 (38.7%)		
			Iatrogenic diabetes mellitus secondary to pancreatectomy	1/12 (8.3%)
Cryptorchidism	Bilateral	4/16 (25.0%)	Azoospermia	3/4¶ (75.0%)
	Monolateral	3/16 (18.9%)	Azoospermia	1/3¶ (33.3%)

†: nephrocalcinosis, multicystic kidney, solitary kidney. ‡: Five of the 6 patients born by obstructed labour were macrosomic fetus. §: Of the 12 patients affected by neonatal hyperinsulinism, 2 had drug resistant epilepsy and 1 had absence seizure, right ear neurosensory deafness and psychiatric intermittent explosive disorder. These three patients also suffered from perinatal hypoxic-ischemic encephalopathy therefore neurological disorders listed above could be consequences either of neonatal hypoglycemia or neonatal hypoxic-ischemic encephalopathy as well. ¶: three males were never tested for fertility nor tried to conceive.

Table 4 — Data of patients with tumors.

Patient ID	Tumor type	Malignant (M) or benign (B)	Genotype	Age at tumor diagnosis	Diagnosis modalities	Surgery	Medical Therapy	Current age
#7	Wilms' tumor	M	GoM IC1	10 y	US Abdominal screening	Nephrectomy	-	19 y
#15	Early T-acute lymphoblastic leukemia	M	LoM-IC2, IC2 deletion	21 y	Hematological screening in myelodysplasia	Splenectomy due to graft failure	AEIOP-BFM ALL 2009 protocol, Allogeneic Bone Marrow Transplantation	Died at 23 y after relapses
#18	Wilms' tumor	M	UPD(11) pat	2 y	US Abdominal screening	Nephrectomy	-	42 y
#20	Wilms' tumor	M	GoM IC1 (microdeletion) [39]	5 y	Hematuria	Nephrectomy	AIEOP TW 2003 protocol	18 y
#23	Hepatoblastoma, cholangioblastic variant	M	LoM-IC2	22 y	Abdominal mass	Orthotopic liver transplant + pancreatic-duodenectomy	Adjuvant chemotherapy	Died at 27 y after relapses
#29	Intratubular Germ Cell Neoplasia, Unclassified	M	*CDKN1C* mut	27 y	Testicular mass	Orchidectomy	-	31 y

#33	Wilms' tumor	M	UPD(11) pat	3 y	Abdominal US due to intercurrent pathology	Renal Lobectomy	Adjuvant chemotherapy	18 y
#21	Testicular Sertoli-cell tumor	M	Not performed	24 y	Testicular mass	Orchidectomy	-	41 y
	Non-functional adrenal adenoma	B with uncertain malignant potential		22 y	Incidentaloma at US	Laparoscopic adrenalectomy	-	
#4	Uterine myoma	B	Negative	40 y	Menorrhagia	Myomectomy	-	58 y
#5	Mammary fibroepithelioma	B	Negative	16 y	Tenderness, self-examination	Tumorectomy	-	18 y
#22	Hepatic angioma	B	Negative	6y	US abdominal screening	-	-	19 y
	Mammary fibroepithelioma	B		18 y	Tenderness, self-examination	-	-	19 y

Chapter 6.

FUTURE PERSPECTIVES

Since the birth of Louise Brown in 1978, continuous improvements in the various therapies and methods have led to a growing success of ART. The EIM Consortium (European IVF-monitoring Consortium), from 1997 to 2014, has reported on more than 8 million treatments (8 010 527) leading to the birth of nearly 1.5 million infants (1 478 452). In our country the rate of ART infants per national births has raised from 1.2% in 2007 [de Mouzon J. et al. 2007] to 2.2% in 2014, according to the last report of ESHRE Society [De Geyter C. et al. 2014]. In the face of growing demand for assisted reproductive technology, the potential health impact on women and babies who are conceived through ART remains a still unresolved concern. After having studied and demonstrated the correlation risk between ART and BWS, we were interested in understanding the relevance and incidence of ART pregnancies in the most important Neonatology Unit of our city.

Since January 2018 we are performing an observational study on newborns conceived through ART and born in the two Neonatology Units of S. Anna Hospital. To date we have recruited 200 newborns conceived through every type of ART: first level such as ovarian stimulation (OS) and intrauterine insemination (IUI), second level as in vitro fertilization-embryo transfer (IVF-ET) and intracytoplasmic sperm injection (ICSI). We considered both homologous and heterologous ART pregnancies. Women were interviewed about their and partner medical status, the past obstetric history, and obviously about ART procedure and pregnancy course. Informations were completed through medical records. Babies' neonatal auxologic parameters were gathered by medical records.

Informed consent was collected and the study received approval from Ethics Committee.

Our cohort encountered 157 pregnancies leading to 200 livebirths. Of these 157 pregnancies, 41 were bigemina, 1 trigemina and 115 were singleton pregnancies. Regarding ART procedures, 82 were FIVET (52.2 %), 59 ICSI (37.5%), 11 IUI (7.0%) and 5 (3.1%) obtained by ovarian stimulation. In the group of ICSI only 18 ART pregnancies (31%) were implemented because of azoospermia although azoospermia should be the only indication for this procedure. Forty pregnancies out of 157 (25.4%)

were obtained by heterologous ART procedures: 31/40 (77.5%) through egg donation, 9/40 (22.5%) through sperm donation and 1/40 (2.5%) through embryo-donation. In FIVET and ICSI procedures, embryo transfer was performed at 3 days in 49 cases (34.7%), at day 5 in 90 couples (63.8%). Transfer was performed with fresh embryos in 79 cases (56.0%), while frozen embryos were used in 59 couples (41.8%). Considering FIVET and ICSI pregnancies (141), in 65 cases only 1 embryo was transferred (46%), in 72 cases 2 embryos were transferred (51%). Of these 72 cases, in 32 couples implantation was successful for only 1 embryo (44.4%). In the 40 ART cycles (55.5%) in which both embryos' implantation was successful, only 31 pregnancies proceeded as bigemina because of spontaneous miscarriage of one of the fetus in the first trimester (8 cases, 20%), and embryo-reduction in one case. Four pregnancies were characterized by transfer of 3 embryos (2.8%) : in 2 cases only 1 embryo implanted and one case was characterized by both spontaneous miscarriage and embryo-reduction of two embryos. Only in one ART pregnancy 4 embryos were transferred but implantation was successful for only one. In the IUI procedures, 2 were bigemina (18.1%), 1 began as quadrigemina (9%) but 1 embryo was lost spontaneously and 1 after embryo-reduction, 8 were singletons pregnancies (72.7%) but in one case became a monoamniotic twin pregnancy. Pregnancies due to ovarian stimulation were singletons in 2 cases (40%) and bigemina in 3 (60%).

Excluding ART pregnancies due to OS and sperm donation (143 cases), testicular sperm extraction (TESE) was performed in 10 cases (6.9%): 6 cases because of diagnosis of azoospermia, in 1 case because of ejaculatory ducts obstruction and in 2 cases without defined medical indication.

In our cohort maternal mean age is 37.33 years (ranging from 24 to 52 years) while paternal average age is 40.35 years (ranging from 28 to 60 years). Twenty-seven out of 157 mothers (17.1%) present a least one morbidity other than infertility, 19 women (12.1%) were affected by two or more chronic diseases. Regarding paternal health, 16 out of 148 (10.8%) men of our cohort present al least one morbidity, no information was available in heterologous pregnancies.

Regarding to obstetric anamnesis, 21 couples experienced spontaneous miscarriage before ART (13.3%), Eight couples experience previous ART cycle, only in 2 cases (25%) leading to a live birth: 4 were unsuccessful (50%) while in 2 cases (25%)

pregnancy was interrupted on parental decision because of diagnosis of multiple congenital defects. Five couples (3.1%) had children naturally conceived but in 3 cases they died: one for cerebral tumor, one at five days of life for severe abdominal malformation and one at the end of the third trimester. One of the two livebirth naturally conceived child is affected by trisomy of chromosome 21.

In our cohort infertility was of maternal origin in 55/157 cases (35%), of paternal origin in 38/157 cases (24.2%), in 48/157 undetermined (30.5%), in 10/157 both of maternal and paternal origin (6.3%). Finally 5 of 157 (3.1%) pregnancies were characterized by absence of male partner and 1 (0.6%) in a fertile couple (they have 3 spontaneous conceived children) to "conceive faster". In our cohort median gestational age is 36,203 weeks (range from 24,572 to 41,715 weeks of gestation). Incidence of preterm birth is 29.9% (47 out of 157). According to World Health Organization 2018 database, this value is the double of the incidences of preterm birth in the 10 countries with the highest rates all over the world (Comorso, Congo, Zimbabwe, Equatorail Guinea, Mozambique, Gabon, Pakistan, Indonesia, Mauritania). Regarding to our country, the incidence has been valued to be 10%, according to the recent (2015-2017) report of SIN Neonatal Network; although this rate includes both ART and spontaneous conceived newborns.

Stratifying by preterm classification, we have 59.5% (28 out of 47) late preterm births, 21.2% of moderately (10 out of 47) preterm births, 12.7% of very preterm births (6 out of 47) and 6.3% of extremely preterm births (3 out 47). In 93 cases out of 157 pregnancies (59.2%), mothers presented almost a gestational complication, in 27 cases (17.1%) two or more.

Regarding to post-partum period, 6 mothers presented severe complications (3.8%) and needed intensive cares: 5 for post-partum hemorrhage and 1 for severe hypertension.

Cesarian section rate is 52.2% (82/157) while spontaneous vaginal delivery rate is 39.4% (62/157) and operative vaginal delivery one is 6.3% (10/157).

Regarding neonatal measures, the average weight is 2.65 kg, average length is 47.5 cm (-0.10 SDS) and average head circumference is 32.7 cm (-0.01 SDS). Incidence of low-weight newborns is 31.5% (63/200), of very low birth weight (VLBW) is 2.5% (5/200) and of extremely low birth weigth (ELBW) is 4.5% (9/200). In 3% of cases (6/200)

neonatal weight was more than 4.0 kg. Seventy babies out of two hundred (35%) needed medical care in intensive care

These are preliminary data: we would like to increase the cohort and compare these data with a control group of naturally conceived newborns. We would like to highlight any significant differences in the incidence of preterm birth, malformations, SGA or LGA newborns and obstetric complications between ART and spontaneous pregnancies. We would also like to compare different subgroups in ART pregnancies: there are reports of correlation between fresh embryos and greater incidence of low birth weight while the use of frozen embryos would correlate with higher risk of LGA newborns [Dunietz GL et al. 2017, Pinborg A. et al. 2014]; even the day of the transfer would seem to affect gestational age and birth weight [Wang YA et al 2011, Wang X et al. 2017].

A further ongoing objective is the follow-up of children up to at least two years of age by evaluating parameters of growth and neuromotor development. The real success of assisted reproduction techniques should be measured in terms of the birth of healthy individuals even in the long term. The association between the use of ART and autism spectrum disorder (ASD) [Liu L et al. 2017] or epilepsy [Kettener LO et al. 2017] risk in offspring has been explored in several studies, but the result is still inconclusive. We would like to carry out a follow-up of at least 2 years for all the cases in our study by monitoring through telephone interviews and visits to growth parameters and stages of neuropsychomotor development

Finally we would like to carry out a methyloma analysis of two specific subgroups: children with malformations and SGA newborns. It is increasingly evident in literature that ART leads to epigenetic modifications. The most valid hypothesis is that the various epigenetic alterations even at the level of the imprinted genes constitute a summation of effects due to the inheritance of pathologies transmitted by gametes, and therefore also due to sterility in itself, to reproduction in old age, to hyperstimulation, to ART with all its technologies, to the means of cultivation and to the environment of the same [Hiura H. et al. 2014]. Defining DNA methylation patterns in the early human embryo coinceived through ART is a plea for a more careful approach to this technology.

CONCLUSIVE REMARKS

In the last years, in collaboration with national and international prestigious centers, we had the opportunity to broaden and deepen some clinical and molecular aspects of Beckwith-Wedemann spectrum. An extreme clinical variability characterizes this condition underlying the necessity of a multidisciplinary approach to the patients. This clinical heterogeneity represents a challenge for the clinician but making diagnosis implies stress for tumor surveillance for patients and their families. So defining certain clinical diagnostic criteria is a priority for clinicians. Also understand genotype-phenotype correlations, in order to outline targeted sub-groups follow up strategies, is fundamental. The expansion in understanding the biochemical complexity of BWSp, together with the availability of new sophisticated molecular technologies, will lead to deepen the molecular pathogenetic mechanisms of BWSp and to better define the clinical implications and the follow up strategies for these congenital disorders.

REFERENCES

Alders M. et al. **Determination of KCNQ1OT1 and H19 methylation levels in BWS and SRS patients using methylation-sensitive high-resolution melting analysis**. Eur J Hum Genet 2009: 17: 467– 473.

Aleck KA, Hadro TA. **Dominant inheritance of Wiedemann-Beckwith syndrome: further evidence for transmission of "unstable premutation" through carrier women**. Am J Med Genet. 1989;33:155–60

Angiolini E. et al. **Developmental adaptations to increased fetal nutrient demand in mouse genetic models of Igf2-mediated overgrowth**. FASEB J 2011: 25: 1737– 1745.

Azzi, S. et al. **Multilocus methylation analysis in a large cohort of 11p15-related foetal growth disorders (Russell Silver and Beckwith Wiedemann syndromes) reveals simultaneous loss of methylation at paternal and maternal imprinted loc**i Hum Mol Genet, 18 (2009), pp. 4724-4733

Beckwith JB. **Extreme cytomegaly of the adrenal fetal cortex, omphalocele, hyperplasia of kidneys and pancreas, and Leydig-cell hyperplasia: Another syndrome**. West Soc Pediatr Res. 1963; 115.

Bémurat L, et al. **Successful laparoscopic operation of bilateral pheochromocytoma in a patient with Beckwith-Wiedemann syndrome**. J Hum Hypertens. 2002;16:281–4

Bertino E. et al. **Neonatal anthropometric charts: the Italian neonatal study compared with other European studies. J Pediatr Gastroenterol Nutr** 2010: 51: 353– 361.

Bertino E. et al. **Auxological evaluation of newborns**. J Biol Regul Homeost Agents 2012: 26 (3 Suppl): 15– 17.

Bertoin F. et al. **Genome-wide paternal uniparental disomy as a cause of Beckwith-Wiedemann syndrome associated with recurrent virilizing adrenocortical tumors**. Horm Metab Res, 47 (2015), pp. 497-503

Bessho F. et al. **Mass screening in Japan increased the detection of infants with neuroblastoma without a decrease in cases in older children** J Pediatr, 119 (1991), pp. 237-241

Bliek J et al. **Increased tumour risk for BWS patients correlates with aberrant H19 and not KCNQ1OT1 methylation: occurrence of KCNQ1OT1 hypomethylation in familial cases of BWS** Hum Mol Genet, 10 (2001), pp. 467-476

Bliek J. et al: **Epigenotyping as a tool for the prediction of tumor risk and tumor type in patients with Beckwith-Wiedemann syndrome (BWS).** J Pediatr 2004; **145**: 796–799.

Bliek, J. et al. **Hypomethylation at multiple maternally methylated imprinted regions including PLAGL1 and GNAS loci in Beckwith-Wiedemann syndrome.** Eur J Hum Genet 2009: 17: 611– 619.

Blohm M.E. et al. **Alpha 1- fetoprotein (AFP) reference values in infants up to 2 years of age.** Pediatr. Hematol. Oncol. 1998 15, 135-142

Bowdin S. et al. **A survey of assisted reproductive technology births and imprinting disorders**. Hum Reprod. 2007;22(12):3237–3240

Brioude F. et al. **Beckwith-Wiedemann syndrome: growth pattern and tumor risk according to molecular mechanism, and guidelines for tumor surveillance**. Horm Res Paediatr.(2013

Brioude F. et al. **Mutations of the imprinted CDKN1C gene as a cause of the overgrowth BeckwitheWiedemann syndrome: clinical spectrum and functional characterization**. Hum. Mutat. 2015, 36 (9), 894-902.

Brioude F. et al. **Hypercortisolism due to a Pituitary Adenoma Associated with Beckwith-Wiedemann Syndrome.** Horm Res Paediatr. 2016;86:206–11.

Brioude F. et al. **Expert consensus document: Clinical and molecular diagnosis, screening and management of Beckwith-Wiedemann syndrome: an international consensus statement**. Nat Rev Endocrinol. 2018;14:229–49.

Cacciari E. et al. **Italian cross-sectional growth charts for height, weight and BMI (2 to 20 yr).** J Endocrinol Invest. 2006;29:581–93

Cai X, Cullen BR. **The imprinted H19 noncoding RNA is a primary microRNA precursor**. RNA 2007 13:313–316.

Calvello M. et al. **Quantitative DNA methylation analysis improves epigenotype-phenotype correlations in Beckwith-Wiedemann syndrome**. Epigenetics 2013; **8**: 1053–1060.

Canavese F. et al. **Sperm count of young men surgically treated for cryptorchidism in the first and second year of life: fertility is better in children treated at a younger age.** Eur J Pediatr Surg Off J Austrian Assoc Pediatr Surg Al Z Kinderchir. 2009;19:388–91.

Cappuccio G. et al. **Giant breast tumors in a patient with Beckwith-Wiedemann syndrome**. Am J Med Genet A 2014; 164A: 182–185

Cardarelli L. et al. **Silver-Russell syndrome and Beckwith-Wiedemann syndrome phenotypes associated with 11p duplication in a single family**. Pediatr Dev Pathol 2010, 13:326–330

Caspary T. et al. **Oppositely imprinted genes p57(Kip2) and igf2 interact in a mouse model for Beckwith-Wiedemann syndrome**. Genes Dev 1999: 13: 3115– 3124

Chan E, Wayne C, Nasr A. **FRCSC for Canadian Association of Pediatric Surgeon Evidence-Based Resource. Ideal timing of orchiopexy: a systematic review**. Pediatr Surg Int. 2014;30:87–97

Chang AS. et al. **Association between Beckwith-Wiedemann syndrome and assisted reproductive technology: a case series of 19 patients**. Fertil Steril. 2005;83(2):349–354

Chen Z et al. **Large offspring syndrome: a bovine model for the human loss-of-imprinting overgrowth syndrome Beckwith-Wiedemann**. Epigenetics. 2013;8(6):591–601

Chen Z. et al. **Characterization of global loss of imprinting in fetal overgrowth syndrome induced by assisted reproduction.** Proc Natl Acad Sci USA. 2015;112(15):4618–4623

Chiesa N. et al. **The KCNQ1OT1 imprinting control region and non-coding RNA: new properties derived from the study of Beckwith-Wiedemann syndrome and Silver-Russell syndrome cases**. Hum Mol Genet. 2012;21:10–25.

Choufani S et al. **Molecular findings in Beckwith-Wiedemann syndrome**. Am J Med Genet C Semin Med Genet 2013, 163C:13-140

Clericuzio et al**. Serum alpha-fetoprotein screening for hepatoblastoma in children with Beckwith-Wiedemann syndrome or isolated hemihyperplasia**. J Pediatr, 143 (2003), pp. 270-272

Clouston WM et al. **Virilizing adrenal adenoma in an adult with the Beckwith-Wiedemann syndrome: paradoxical response to dexamethasone**. Clin Endocrinol (Oxf). 1989;31:467–73.

Cohen MM Jr. **Beckwith-Wiedemann syndrome: historical, clinicopathological, and etiopathogenetic perspectives**. Pediatr Dev Pathol 2005; 8: 287–304.

Cordeiro A. et al. **Relevance of genomic imprinting in intrauterine human growth expression of CDKN1C, H19, IGF2, KCNQ1 and PHLDA2 imprinted genes.** J Assist Reprod Genet 2014: 31: 1361– 1368.

Cooper, W.N. et al. **Molecular subtypes and phenotypic expression of Beckwith-Wiedemann syndrome.** Eur. J. Hum. Genet. 2005 13, 1025-1032

Custódio G et al. **Impact of neonatal screening and surveillance for the TP53 R337H mutation on early detection of childhood adrenocortical tumors**. J Clin Oncol, 31 (2013), pp. 2619-262

DeBaun, M.R et al. **Nephromegaly in infancy and early childhood: a risk factor for Wilms tumor in BeckwitheWiedemann syndrome**. J. Pediatr. 1998, 132, 401-404

DeBaun MR et al. **Epigenetic alterations of H19 and LIT1 distinguish patients with Beckwith-Wiedemann syndrome with cancer and birth defects**. Am J Hum Genet, 70 2002, pp. 604-611

DeBaun MR et al. **Association of in vitro fertilization with Beckwith-Wiedemann syndrome and epigenetic alterations of LIT1 and H19**. Am J Hum Genet. 2003;72(1):156–160

De Geyter C et al. **European IVF-monitoring Consortium (EIM) for the European Society of Human Reproduction and Embryology (ESHRE). ART in Europe, 2014: results generated from European registries by ESHRE: The European IVF-monitoring Consortium (EIM) for the European Society of Human Reproduction and Embryology (ESHRE).** Hum Reprod. 2018 Sep 1;33(9):1586-1601

de Mouzon J et al. **European IVF-Monitoring (EIM) Consortium, for the European Society of Human Reproduction and Embryology (ESHRE). Assisted reproductive technology in Europe, 2006: results generated from European registers by ESHRE**. Hum Reprod. 2010;25(8):1851–186

de Mouzon J. et al. **European IVF-Monitoring (EIM); Consortium for the European Society on Human Reproduction and Embryology (ESHRE). Assisted reproductive technology in Europe, 2007: results generated from European registers by ESHRE**. Hum Reprod. 2012;27(4):954–966

de Waal E. et al. **The cumulative effect of assisted reproduction procedures on placental development and epigenetic perturbations in a mouse model**. Hum Mol Genet. 2015;24(24):6975–6985

Dhont M et al. **Perinatal outcome of pregnancies after assisted reproduction: a case-control study**. Am J Obstet Gynecol. 1999;181(3):688–69

Diaz-Meyer N. et al. **Silencing of CDKN1C (p57KIP2) is associated with hypomethylation at KvDMR1 in Beckwith-Wiedemann syndrome**. J Med Genet 2003: 40: 797– 80

Doornbos ME, et al. **Infertility, assisted reproduction technologies and imprinting disturbances: a Dutch study.** Hum Reprod. 2007;22(9):2476–2480

Dunietz GL et al. **Assisted Reproductive Technology and Newborn Size in Singletons Resulting from Fresh and Cryopreserved Embryos Transfer.** PLoS One. 2017 Jan 23;12(1)

Eggermann T. **Russell-Silver syndrome**. Am J Med Genet Part C 2010, 154C:355–364.

Eggerman T et al. **Clinical utility gene card for Beckwith- Wiedemann syndrome** . Eur J Hum Genet 2014; 22

Eggermann K. et al. **EMQN best practice guidelines for the molecular genetic testing and reporting of chromosome 11p15 imprinting disorders: Silver-Russell and Beckwith-Wiedemann syndrome.** Eur J Hum Genet. 2016;24(10):1377–1387

Elliott M. et al. **Clinical features and natural history of Beckwith-Wiedemann syndrome: presentation of 74 new cases**. Clin Genet, 46:168–174 (1994)

Engel JR et al. **Epigenotype-phenotype correlations in Beckwith-Wiedemann syndrome**. J Med Genet, 37 (2000), pp. 921-926

Feyles F. et al. **Improved sperm count and motility in young men surgically treated for cryptorchidism in the first year of life.** Eur J Pediatr Surg Off J Austrian Assoc Pediatr Surg Al Z Kinderchir. 2014;24:376–80

Ferraretti AP. et al. **European IVF-Monitoring (EIM); Consortium for European Society of Human Reproduction and Embryology (ESHRE). Assisted reproductive technology in Europe, 2008: results generated from European registers by ESHRE**. Hum Reprod. 2012;27(9):2571–2584

Ferraretti AP. et al. **European IVF-Monitoring (EIM) Consortium for the European Society of Human Reproduction and Embryology (ESHRE). Assisted reproductive technology in Europe, 2009: results generated from European registers by ESHRE**. Hum Reprod. 2013;28(9):2318–2331

Fleisher AS, Meltzer SJ, James SP. **Colon polyps in Beckwith-Wiedmann syndrome: role of imprinted genes**. Gastroenterology. 2000;118:637

Gardiner K. et al. **Brain abnormalities in patients with BeckwitheWiedemann syndrome**. Am. J. Med. Genet. A 2012, 158, 1388-1394.

Gaston V. et al: **Analysis of the methylation status of the KCNQ1OT and H19 genes in leukocyte DNA for the diagnosis and prognosis of Beckwith-Wiedemann syndrome**. Eur J Hum Genet 2001, 9:409–418.

Gianotten J et al. **Chromosomal region 11p15 is associated with male factor subfertility.** Mol Hum Reprod. 2003;9:587–92.

Gicquel C et al. **In vitro fertilization may increase the risk of Beckwith-Wiedemann syndrome related to the abnormal imprinting of the KCN1OT gene**. Am J Hum Genet. 2003;72(5):1338–1341

Goldman M. et al. **Renal abnormalities in BeckwitheWiedemann syndrome are associated with 11p15.5 uniparental disomy.** J. Am. Soc. Nephrol. 2002, 13, 2077-2084.

Gomes MV et al. **Abnormal methylation at the KvDMR1 imprinting control region in clinically normal children conceived by assisted reproductive technologies**. Mol Hum Reprod. 2009;15(8):471–477

Greer KJ et al. **Beckwith-Wiedemann syndrome in adults: observations from one family and recommendations for care**. Am J Med Genet A. 2008;146A:1707–12

Guo L. et al. **Altered gene expression and methylation of the human chromosome 11 imprinted region in small for gestational age (SGA) placentae**. Dev Biol 2008, 320:79–91.

Gurrieri, F. et al. **Mild Beckwith-Wiedemann and severe long-QT syndrome due to deletion of the imprinting center 2 on chromosome 11p.** Eur. J. Hum. Genet. 2013, 21, 965-969.

Halliday J. et al. **Beckwith-Wiedemann syndrome and IVF: a case-control study**. Am J Hum Genet 2004; **75**: 526–528.

Hansen M et al. **Assisted reproductive technology and birth defects: a systematic review and meta-analysis**. Hum Reprod Update. 2013;19(4):330–353

Hao Y. et al. **Tumour-suppressor activity of H19 RNA**. Nature 1993, 365:764–767

Hark AT. et al. **CTCF mediates methylation-sensitive enhancer- blocking activity at the H19/Igf2 locus**. Nature 2000, 405:486–489.

Hart R, Norman RJ. **The longer-term health outcomes for children born as a result of IVF treatment: part I–general health outcomes.** Hum Reprod Update. 2013;19(3):232–243

Hatada I. et al**. An imprinted gene p57KIP2 is mutated in Beckwith-Wiedemann syndrome**. Nat Genet. 1996; 14:171–173.

Hayward NK et al. **Generation of homozygosity at the c-Ha-ras-1 locus on chromosome 11p in an adrenal adenoma from an adult with Wiedemann-Beckwith syndrome**. Cancer Genet Cytogenet. 1988;30:127–32.

Hedborg F. et al. **The cell type-specific IGF2 expression during early human development correlates to the pattern of overgrowth and neoplasia in the Beckwith-Wiedemann syndrome**. Am J Pathol 1994: 145: 802– 817.

Henry I. et al. **Uniparental paternal disomy in a genetic cancer-predisposing syndrome**. Nature. 1991; 351:665–667.

Hiura H. et al: **Characterization of DNA methylation errors in patients with imprinting disorders conceived by assisted reproduction technologies.** Hum Reprod 2012; 27: 2541–2548.

Hiura H. et al**: Imprinting methylation errors in ART**. Reprod Med Biol. 2014;13(4):193-202.

Hopsu E. et al. **Progressive stapedial fixation in Beckwith-Wiedemann syndrome**. Arch Otolaryngol Head Neck Surg. 2003;129:1131–4

Hori N. et al. **Aberrant CpG methylation of the imprinting control region KvDMR1 detected in assisted reproductive technology-produced calves and pathogenesis of large offspring syndrome**. Anim Reprod Sci. 2010;122(3–4):303–312

Houtenbos I. Ossenkoppele GJ. **Acute myeloid leukemia in a 23-year-old patient with Beckwith-Wiedemann syndrome.** Cancer Genet Cytogenet. 2002;136:90–1.

Ibrahim A. et al. **Methylation analysis and diagnostics of Beckwith-Wiedemann syndrome in 1,000 subjects.** Clin Epigenetics 2014: 6 (1): 11

Inbar-Feigenberg M. et al: **Mosaicism for genome-wide paternal uniparental disomy with features of multiple imprinting disorders: diagnostic and management issues**. Am J Med Genet A 2013; 161A: 13–20.

Itoh N. et al.: **Proportion of cells with paternal 11p15 uniparental disomy correlates with organ enlargement in Wiedemann-beckwith syndrome**. Am J Med Genet 2000; **92**: 111–116.

Jackson RA et al.. **Perinatal outcomes in singletons following in vitro fertilization: a meta-analysis.** Obstet Gynecol. 2004;103(3):551–563

Kagan KO et al. **Novel fetal and maternal sonographic findings in confirmed cases of Beckwith-Wiedemann syndrome**. Prenat Diagn 2015: 35: 394– 399

Kalish JM et al: **Clinical features of three girls with mosaic genome-wide paternal uniparental isodisomy**. Am J Med Genet A 2013; 161A: 1929–1939

Kalish JM et al: **Nomenclature and definition in asymmetric regional body overgrowth**. Am J Med Genet A. 2017 Jul;173(7):1735-1738.

Källén B. et al. **In vitro fertilization (IVF) in Sweden: risk for congenital malformations after different IVF methods.** Birth Defects Res A Clin Mol Teratol. 2005;73(3):162–169

Kanayama N et al. **Deficiency in p57Kip2 expression induces preeclampsia-like symptoms in mice.** Mol Hum Reprod 2002: 8: 1129– 1135.

Kettner LO et al. **Fertility Treatment and Childhood Epilepsy: A Nationwide Cohort Study** Epidemiology. 2017 May;28(3):412-418

Keniry A. et al. **The H19 lincRNA is a developmental reservoir of miR-675 that suppresses growth and Igf1r.** Nat Cell Biol 2012, 14:659–665.

Kramer BS et al. **Cancer screening** Hematol Oncol Clin North Am, 14 (2000), pp. 831-848

Kulkarni R. et al. **Severe intrarenal fibrosis, infundibular stenosis, renal cysts, and persistent perilobar nephrogenic rests in a patient with Beckwith-Wiedemann syndrome 27 years after diffuse nephroblastomatosis and Wilms tumor: natural progression or a consequence of treatment**? J Pediatr Hematol Oncol. 2002;24:389–93

Kunze J, Wiedemann HR. **Wiedemann- Beckwith syndrome**. Ergeb Inn Med Kinderheilkd 1993,61:303–338

Kupka MS et al. **European IVF-Monitoring Consortium, for the European Society of Human Reproduction and Embryology. Assisted reproductive technology in Europe, 2010: results generated from European registers by ESHRE**. Hum Reprod. 2014;29(10):2099–2113

Lapunzina P **Risk of tumorigenesis in overgrowth syndromes: a comprehensive review**. Am J Med Genet C Semin Med Genet, 137C (2005), pp. 53-71

Leighton PA. et al. **Disruption of imprinting caused by deletion of the H19 gene region in mice**. Nature 1995 375: 34–39

Lennerz JK. et al. **Addition of H19 'loss of methylation testing' for Beckwith-Wiedemann syndrome (BWS) increases the diagnostic yield**. J Mol Diagn. 2010;12(5):576–588

Li M, et al. **Imprinting status of 11p15 genes in Beckwith-Wiedemann syndrome patients with CDKN1C mutations**. Genomics 2001, 74: 370–376.

Lidegaard O. et al. **Imprinting diseases and IVF: Danish National IVF cohort study**. Hum Reprod. 2005;20(4):950–954

Lim D. et al. **Clinical and molecular genetic features of Beckwith-Wiedemann syndrome associated with assisted reproductive technologies**. Hum Reprod. 2009;24(3):741–747

Liu L. et al. **Association between assisted reproductive technology and the risk of autism spectrum disorders in the offspring: a meta-analysis**. Sci Rep. 2017 Apr 7;7:46207.

Maher ER et al. **Epigenetic risks related to assisted reproductive technologies: epigenetics, imprinting, ART and icebergs?** Hum Reprod. 2003;18(12):2508–2511

Matsumoto K. et al. **Sensory changes after tongue reduction for macroglossia**. Oral Surg Oral Med Oral Pathol Oral Radiol. 2014;117:e1-2.

Matsuoka S. et al. **p57KIP2, a structurally distinct member of the p21CIP1 Cdk inhibitor family, is a candidate tumor suppressor gene**. Genes Dev 1995, 9:650–662.

McNeil DE et al. **Screening for Wilms tumor and hepatoblastoma in children with Beckwith-Wiedemann syndromes: a cost-effective model**. Med Pediatr Oncol 2001; 37: 349–356.

Mussa A. et al: **Neonatal hepatoblastoma in a newborn with severe phenotype of Beckwith-Wiedemann syndrome**. Eur J Pediatr 2011; 170: 1407–1411.

Mussa, A et al. **Nephrological findings and genotype-phenotype correlation in Beckwith-Wiedemann syndrome**. Pediatr. Nephrol. 2012, 27, 397e406

Mussa A, Russo S, De Crescenzo A, Chiesa N, Molinatto C, **Prevalence of Beckwith-Wiedemann syndrome in North West of Italy.** Am J Med Genet A. 2013 Oct;161A(10):2481-6.

Mussa A. et al: **Alpha-fetoprotein assay on dried blood spot for hepatoblastoma screening in children with overgrowth-cancer predisposition syndromes**. Pediatr Res 2014; 76: 544–548

Mussa A. et al. **Recommendations of the Scientific Committee of the Italian Beckwith-Wiedemann Syndrome Association on the diagnosis, management and follow-up of the syndrome**. European Journal of Medical Genetics 59 2016, 52-64

Mussa A. et al. **(Epi)genotype-phenotype correlations in Beckwith-Wiedemann syndrome**. Eur J Hum Genet. 2016 Feb;24(2):183-90.

Niemitz, E.L et al. **Children with idiopathic hemihypertrophy and BeckwitheWiedemann syndrome have different constitutional epigenotypes associated with Wilms tumor**. Am. J. Hum. Genet. 2005, 77, 887-891.

Palumbo O et al. **A de novo 11p13 microduplication in a patient with some features invoking Silver-Russell syndrome**. Mol Syndromol. 2014;5(1):11–18

Pettenati, M.J. et al. **WiedemanneBeckwith syndrome: presentation of clinical and cytogenetic data on 22 new cases and review of the literature**. Hum. Genet. 1986, 74, 143-154.

Pinborg A. et al. **Large baby syndrome in singletons born after frozen embryo transfer (FET): is it due to maternal factors or the cryotechnique?** Hum Reprod. 2014 Mar;29(3):618-27.

Porteus, P. et al. **Characteristics and outcome of children with Beckwith-Wiedemann syndrome and Wilms' tumor: a report from the National Wilms Tumor Study Group** J Clin Oncol, 18 (2000), pp. 2026-2031

Prada CE, Zarate YA, Hopkin RJ. **Genetic causes of macroglossia: diagnostic approach**. Pediatrics 2012: 129 (2): e431-437.

Priolo M. et al: **MS-MLPA is a specific and sensitive technique for detecting all chromosome 11p15.5 imprinting defects of BWS and SRS in a single-tube experiment**. Eur J Hum Genet 2008; **16**: 565–571.

Reefhuis J. et al. **National Birth Defects Prevention Study. Assisted reproductive technology and major structural birth defects in the United States.** Hum Reprod. 2009;24(2):360–366

Reik W. et al. **Imprinting mutations in the Beckwith-Wiedemann syndrome suggested by altered imprinting pattern in the IGF2-H19 domain**. Hum Mol Genet. 1995; 4:2379–2385.

Romanelli V et al. **CDKN1C mutations in HELLP/preeclamptic mothers of Beckwith-Wiedemann syndrome (BWS) patients**. Placenta 2009: 30: 551– 554.

Romanelli, V. et al. **CDKN1C (p57(Kip2)) analysis in BeckwitheWiedemann syndrome (BWS) patients: genotype-phenotype correlations, novel mutations, and polymorphisms**. Am. J. Med. Genet. A 2010, 152, 1390-1397

Romanelli V. et al. **Constitutional mosaic genome-wide uniparental disomy due to diploidisation: an unusual cancer-predisposing mechanism**. J Med Genet. 2011;48:212–6.

Rossignol S et al. **The epigenetic imprinting defect of patients with Beckwith-Wiedemann syndrome born after assisted reproductive technology is not restricted to the 11p15 region.** J Med Genet. 2006;43(12):902–907

Rump P et al. **Tumor risk in Beckwith-Wiedemann syndrome: a review and meta-analysis**. Am J Med Genet A 2005; 136: 95-104.

Russo S. et al. **A fluorescent method for detecting low-grade 11patUPD mosaicism in Beckwith-Wiedemann syndrome**. Mol Cell Probes 2003; **17**: 295–299.

Russo S. et al. **A multi-method approach to the molecular diagnosis of overt and borderline 11p15.5 defects underlying Silver-Russell and Beckwith-Wiedemann syndromes**. Clin Epigenetics, 8 (2016), p. 23

Sasaki K. et al. **Japanese and North American/European patients with Beckwith-Wiedemann syndrome have different frequencies of some epigenetic and genetic alterations**. Eur J Hum Genet, 15 (2007), pp. 1205-1210

Scott RH et al. **Surveillance for Wilms tumour In at-risk children: pragmatic recommendations for best practice.** Arch Dis Child, 91 (2006), pp. 995-999

Scott RH. et al. **Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) robustly detects and distinguished 11p15 abnormalities associated with overgrowth and growth retardation**. J Med Genet 2008; 45: 106–113.

Shuman C. et al. **Beckwith-Wiedemann Syndrome**; in Pagon RA, Adam MP, Bird TD, Dolan CR, Fong CT, Stephens K (eds). GeneReviews. Seattle (WA), University of Washington, Seattle; (1993-2013).

Slavotinek, A. et al. **Paternally inherited duplications of 11p15.5 and Beckwith-Wiedemann syndrome.** J. Med. Genet. 1997, 34, 819-826.

Smith AC et al. **Severe presentation of Beckwith-Wiedemann syndrome associated with high levels of constitutional paternal uniparental disomy for chromosome 11p15**. Am J Med Genet A 2007; **143A**: 3010–3015.

Sun FL. et al. **Transactivation of Igf2 in a mouse model of Beckwith-Wiedemann syndrome**. Nature 1997: 389 (6653): 809– 815.

Sutcliffe AG et al. **Minor congenital anomalies, major congenital malformations and development in children conceived from cryopreserved embryos**. Hum Reprod. 1995;10(12):3332–3337

Sutcliffe AG et al. **Assisted reproductive therapies and imprinting disorders—a preliminary British survey.** Hum Reprod 2006; 21: 1009–1011.

Sutcliffe AG et al. **Outcome of assisted reproduction**. Lancet. 2007;370(9584):351–359

Talaulikar VS, Arulkumaran S. **Reproductive outcomes after assisted conception**. Obstet Gynecol Surv. 2012;67(9):566–583

Tee L et al. **Epimutation profiling in Beckwith-Wiedemann syndrome: relationship with assisted reproductive technology**. Clin Epigenetics. 2013;5(1):23

Tomlinson JK, et al. **Long-term outcomes of surgical tongue reduction in Beckwith-Wiedemann syndrome**. Plast Reconstr Surg. 2007;119:992–1002.

Trobaugh-Lotrario AD et al. **Hepatoblastoma in children with Beckwith-Wiedemann syndrome: does it warrant different treatment?** J Pediatr Hematol Oncol, 36 2014, pp. 369-373

Tunste SJ. et al. **Fetal overgrowth in the Cdkn1c mouse model of Beckwith-Wiedemann syndrome.** Dis Model Mech 2011: 4: 814– 821

Valente FM et al. **Transcription alterations of KCNQ1 associated with imprinted methylation defects in the Beckwith-Wiedemann locus**. Genet Med Off J Am Coll Med Genet. 2019

Vals MA et al. **The frequency of methylation abnormalities among Estonian patients selected by clinical diagnostic scoring systems for Silver-Russell Syndrome and Beckwith-Wiedemann Syndrome** Genet Test Mol Biomarkers, 19 (2015), pp. 684-691

Vermeiden JP, Bernardus RE. **Are imprinting disorders more prevalent after human in vitro fertilization or intracytoplasmic sperm injection?** Fertil Steril. 2013;99(3):642–65

Wang YA et al. **Transfers of fresh blastocysts and blastocysts cultured from thawed cleavage embryos are associated with fewer miscarriages**. Reprod Biomed Online. 2011 Dec;23(6):777-88.

Wang X et al. **Comparative neonatal outcomes in singleton births from blastocyst transfers or cleavage-stage embryo transfers: a systematic review and meta-analysis** Reprod Biol Endocrinol. 2017 May 4;15(1):36.

Weksberg R, Shuman C: **Beckwith–Wiedemann syndrome and hemihypertrophy**; In: Cassidy SB, Allanson JE (eds):: Management of Genetic Syndromes, 2nd ed. New York: John Wiley & Sons, Inc, 2000, pp 101–116

Weksberg R. et al: **Tumor development in the Beckwith-Wiedemann syndrome is associated with a variety of constitutional molecular 11p15 alterations including imprinting defects of KCNQ1OT1**. Hum Mol Genet 2001; 10: 2989–3000.

Weksberg R. et al. **Beckwith-Wiedemann syndrome**. Am J Med Genet Part C 2005, 137C:12–23.

Weksberg et al. **Beckwith-Wiedemann syndrome**. Eur J Hum Genet 2010; 18: 8-14

Wiedemann HR. **Familial malformation complex with umbilical hernia and macroglossia - a 'new syndrome'?** J Genet Hum. 1964; 13:223–232.

Williams CL et al. **Cancer risk among children born after assisted conception**. N Engl J Med. 2014;370(10):975–976

WN et al: **Molecular subtypes and phenotypic expression of Beckwith-Wiedemann syndrome**. Eur J Hum Genet; 2005, 13: 1025-1032

Yamamoto K. et al. **Mass screening and age-specific incidence of neuroblastoma in Saitama Prefecture**, **Japan.** J Clin Oncol, 13 (1995), pp. 2033-2038

Young LE et al. **Large offspring syndrome in cattle and sheep**. Rev Reprod. 1998;3(3):155–163

Young LE et al. **Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture**. Nat Genet. 2001;27(2):153–154

Zarate YA, et al: **Experience with hemihyperplasia and Beckwith-Wiedemann syndrome surveillance protocol.** Am J Med Genet A 2009; 149A: 1691–1697.

Zollino M. et al. **A case of Beckwith-Wiedemann syndrome caused by a cryptic 11p15 deletion encompassing the centromeric imprinted domain of the BWS locus**. J Med Genet. 2010;47:429–32.

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