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## POLICY

# EMQN best practice guidelines for the molecular genetic testing and reporting of chromosome 11p15 imprinting disorders: Silver–Russell and Beckwith–Wiedemann syndrome

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Molecular genetic testing for the 11p15-associated imprinting disorders Silver–Russell and Beckwith–Wiedemann syndrome (SRS, BWS) is challenging because of the molecular heterogeneity and complexity of the affected imprinted regions. With the growing knowledge on the molecular basis of these disorders and the demand for molecular testing, it turned out that there is an urgent need for a standardized molecular diagnostic testing and reporting strategy. Based on the results from the first external pilot quality assessment schemes organized by the European Molecular Quality Network (EMQN) in 2014 and in context with activities of the European Network of Imprinting Disorders (EUCID.net) towards a consensus in diagnostics and management of SRS and BWS, best practice guidelines have now been developed. Members of institutions working in the field of SRS and BWS diagnostics were invited to comment, and in the light of their feedback amendments were made. The final document was ratified in the course of an EMQN best practice guideline meeting and is in accordance with the general SRS and BWS consensus guidelines, which are in preparation. These guidelines are based on the knowledge acquired from peer-reviewed and published data, as well as observations of the authors in their practice. However, these guidelines can only provide a snapshot of current knowledge at the time of manuscript submission and readers are advised to keep up with the literature.

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## CLINICAL BACKGROUND

### Silver–Russell syndrome

Silver–Russell syndrome (Russell–Silver syndrome; SRS; OMIM 180860) is mainly defined by severe intrauterine and postnatal growth restriction (<P3) associated with a variable spectrum of further features (for a review see Saal<sup>1</sup>). The typical SRS phenotype includes a relative macrocephaly, a triangular-shaped face with a prominent forehead, body and limb asymmetry, and feeding difficulties (for a review see Azzi *et al*<sup>2</sup> and Wakeling *et al*<sup>3</sup>). Some patients show a mild motor and cognitive developmental delay, including learning

difficulties and speech delay.<sup>3,4</sup> Furthermore, adult follow-up data indicate an increased risk for metabolic disorders in later life.<sup>5</sup>

The clinical presentation is variable and probably partly influenced by the mosaic distribution of molecular changes.<sup>6</sup> Although several scoring systems have been suggested (for a review see Azzi *et al*<sup>7</sup>), the clinical diagnosis of SRS is difficult because of its molecular and clinical heterogeneity (Tables 1 and 2). Therefore, molecular testing is often applied to patients who do not fulfill the strict clinical criteria, resulting in a detection rate lower compared with that expected from studies based on clinically well-defined cohorts.<sup>8</sup>

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**Table 1** Molecular subtypes and their frequencies in the 11p15.5-associated imprinting disorders (frequencies of molecular subtypes are taken from refs 1,6,9)

Chromosome	Type of mutations/epimutation	Frequency	MLID	Mosaicism	
				possible	Recurrence risk
<i>SRS (OMIM 180860)<sup>1,6</sup></i>					
11p15.5	<i>H19/IGF2</i> :IG-DMR hypomethylation	40–60%	7–10%	Yes	Empirically low, only in rare cases increased due to genomic trans-acting mutations (eg, MLID)
	Duplications/deletions	<1%	No	No	Up to 50%, depending on the gene content of the aberration and the sex of the parent contributing the affected allele. In case of duplication of the whole 11p15 region, SRS in case of a maternal and BWS in case of a paternal transmission.
	UPD	1 case	—	Yes	Empirically low
	<i>CDKN1C</i> point mutation	1 family	No	No	0% or 50%, depending on the sex of the parent contributing the affected allele
	<i>IGF2</i> point mutation	1 family	No	No	0 or 50%, depending on the sex of the parent contributing the affected allele
7	upd(7)mat	4–10%	No	No	Empirically low, but some may be high because of familial translocations
	upd(7q)mat	Single cases	No	No	Empirically low
	Duplications/deletions/translocations affecting 7p13 and 7q32	Single cases	No	No	Up to 50%
14q32	upd(14)mat, epimutation, duplications	Single cases	Unknown	yes	Refer to TS14 literature
	Whole genome (Mosaic) maternal unidiploidy (submicroscopic) chromosomal imbalances	Single cases ~1%	No	Yes No	No Up 50%, depending on the chromosome and type of rearrangement
<i>BWS (OMIM130650)<sup>9</sup></i>					
11p15.5	<i>KCNQ1OT1</i> :TSS-DMR hypomethylation	50–60%	25%	Yes	Empirically low, only in some cases increased because of genomic trans-acting mutations (eg, MLID)
	<i>H19/IGF2</i> :IG-DMR hypermethylation	5–10%	No	Yes	20% of patients carry OCT4/SOX2 binding site mutations or deletions within the ICR1. In general, the recurrence risk is empirically low, but if an underlying genetic defect is present it may be up to 50%, depending on the gender of the contributing carrier
	upd(11)pat	20–25%	No	Yes	Empirically low, but some may be high due to translocations
	Duplications/deletions/(translocations)	1–2%	No	No	Up to 50%, depending on the gene content of the aberration and the sex of the parent contributing the affected allele. In case of duplication of the whole 11p15 region, SRS in case of a maternal and BWS in case of a paternal transmission
	<i>CDKN1C</i> mutations	5% (sporadic) 50% (familial)	No	No	0% or 50%, depending on the sex of the parent contributing the affected allele
Whole genome	(Mosaic) paternal unidiploidy	~1%?		Yes	No

Abbreviations: BWS, Beckwith–Wiedemann syndrome; MLID, multilocus imprinting disturbance; SRS, Silver–Russell syndrome.

### Beckwith–Wiedemann syndrome

Beckwith–Wiedemann syndrome (BWS; OMIM130650<sup>9</sup>) was initially called EMG syndrome from its three main features exomphalos/umbilical hernia, macroglossia and (neonatal) gigantism. Additional signs include neonatal hypoglycemia, hemihypertrophy, organomegaly (essentially kidney, liver, spleen, pancreas), earlobe creases, polyhydramnios, hemangioma and cardiomyopathy. In 5–7% of children, embryonal tumors (most commonly Wilm's tumor (WT)) are diagnosed (for a review see Brioude *et al*<sup>10</sup>). The clinical diagnosis of BWS is often difficult because of its variable presentation and overlap with other overgrowth syndromes (for a review see Shuman *et al*<sup>11</sup>), and hence several scoring systems have been suggested<sup>12,13</sup> (reviewed by Ibrahim *et al*<sup>14</sup>). Robust genotype/epigenotype–phenotype correlations have been established<sup>10,14–16</sup> (Table 1): hemihypertrophy is strongly associated with upd(11)pat, exomphalos with *H19/IGF2*:IG-DMR hypermethylation and *CDKN1C* mutations, and, most

importantly, the risk for WT is predominant in *H19/IGF2*:IG-DMR hypermethylation and upd(11)pat. By contrast, other embryonal tumors such as hepatoblastoma, neuroblastoma and adrenal tumors are observed in patients with *KCNQ1OT1*:TSS-DMR or upd(11)pat, although at a much lower incidence. In patients with a mosaic paternal uniparental diploidy (also named 'genome-wide UPD'), further tumors can occur, with a later age of onset than in case of the other molecular subtypes. Hence, the determination of the molecular subtype is important for an individual prognosis and management. Nevertheless, phenotypic transitions are fluid, ranging from some features that include 'incomplete BWS' to the full clinical picture. Thus, molecular testing for BWS is also suggested in case of isolated hemihypertrophy as molecular changes in 11p15.5 have also been identified in patients with isolated body asymmetry. It should furthermore be considered in bilateral WT, familial WT cases and WT patients with at least one feature of BWS.

**Table 2 Overview on the currently applied methods for 11p15.5 testing and their limitations**

Method	Suitability to detect					Suitable for		Average amount of DNA needed	Limitations
	Loci per test	UPD	Epimutation	CNV	SNP	prenatal testing	postnatal testing		
<i>MS methods based on methylation-sensitive enzymes</i>									
MS Southern blot	1	Y	Y	Y	N	(Y)		5 µg	Large amounts of DNA, time-consuming. Main limitation is restriction site interrogation of epimutation at single CpGs within the DMRs Sensitivity is relatively low in comparison with the other techniques. No commercially available kit Only indirect discrimination of CNVs. In case of a positive result, further testing is required to discriminate the underlying change Commercial kits. May have reduced sensitivity for detection of mosaic epimutation and UPD. SNVs can interfere. Dependent on the probe composition of the kit, a discrimination between the types of (epi)mutations and UPD can be possible
MS-MLPA	Up to ~46 <sup>a</sup>	Y	Y	Y	N	Y		50–100 ng	
<i>MS methods based on bisulfite treatment: these methods are not MS in sensu stricto, as they are based on primers equally amplifying unmethylated or methylated alleles</i>									
MS PCR	1	Y	Y	Y	N	Y		1 µg	No commercially available kit. Only indirect discrimination of CNVs. In case of a positive result, further testing is required to discriminate the underlying change No commercially available kit.
ASMM RTQ-PCR	1	Y	Y	N	N	Y		150 ng	Only indirect discrimination of CNVs. In case of a positive result, further testing is required to discriminate the underlying change Does not examine discrete CpG sites but looks at regional profile. No commercially available kit
MS-HRMA	1	Y	Y	Y	Y	Y		1 µg	Only indirect discrimination of CNVs. In case of a positive result, further testing is required to discriminate the underlying change
MS pyrosequencing	1	N	Y	Y	(Y)	Y		> 100 ng	Only indirect discrimination of CNVs. In case of a positive result, further testing is required to discriminate the underlying change May have reduced sensitivity for detection of mosaic epimutation. Read-length limits analyzed CpGs per read. Only indirect discrimination of CNVs.
MS-SNuPE	Up to 10	Y	Y	Y	N	Y		100 ng	In case of a positive result, further testing is required to discriminate the underlying change No commercially available kit. Only indirect discrimination of CNVs. In case of a positive result, further testing is required to discriminate the underlying change
<i>Non-MS tests for CNV and/or UPD detection</i>									
Microsatellite analysis (STR)	1	Y	N	Y	N	Y		<20 ng per locus	DNA of at least one parent required; restricted to the detection of CNVs and UPD
qPCR	1	N	N	Y	N	Y		200 ng	Only unbalanced alterations are detected. Flexible design targeting the region of interest; small CNVs can be detected
Molecular karyotyping (arrayCGH, SNP array)	Whole genome: depends on resolution	Y (SNP) N (CGH)	N	Y	N	Y		250 ng	Only unbalanced alterations are detected Does not detect epimutations. No UPD detection by arrayCGH. Uneven probe coverage can leave CNVs undetected
DNA sequencing	Locus-specific	N	N	N	Y	Y		Depending on the size of the gene: ~ 60–200 ng	Only sequencing variants can be detected, other genomic variants including CNVs escape detection. In case of homozygosity of SNP large deletions should be considered

Abbreviations: ASMM RTQ-PCR, allele-specific methylated multiplex real-time quantitative PCR; CHG, comparative genomic hybridization; CNV, copy number variation (deletions/duplications); MLPA, multiplex ligation probe-dependent amplification; MS, methylation-specific; MS-HRMA, MS-high-resolution melting analysis; MS-SNuPE, methylation-specific single-nucleotide primer extension; N, no; qPCR, quantitative PCR; SNP, single nucleotide variation – monogenic disease-causing variant; UPD, uniparental disomy; Y, yes.

<sup>a</sup>The number of 46 probes refers to the conventional MLPA kits.

## THE CHROMOSOMAL REGION 11P15.5

One of the major imprinted regions in humans is localized to the short arm of chromosome 11 (11p15.5) (Figure 1). Several of the 11p15 genes are involved in human growth and development, as well as in tumorigenesis. As a result, SRS and BWS, as the two imprinting disorders (IDs) associated with 11p15.5 alterations, are clinically characterized by disturbed growth and, in the case of BWS, associated with an increased risk for tumors.

The chromosomal region 11p15.5 spans ~1 Mb and harbors two separate imprinting control regions (ICRs): the chromosomal region 11p15.5 spans ~1 Mb and harbors two separate ICRs (Supplementary Table 1): the telomeric one, ICR1, includes the *H19/IGF2:IG-DMR*, which is methylated on the paternal allele, whereas the centromeric one, ICR2, includes the *KCNQ1OT1:TSS-DMR*, which is maternally methylated.

In the telomeric ICR1 a different chromatin architecture of the parental alleles leads to a reciprocal expression of *H19* and *IGF2* (insulin-like growth factor 2). The ICR1 contains seven CTCF (CCCTC-binding factor) target sites in the DMR, which is located 2 kb upstream of *H19*. The zinc-finger binding factor CTCF binds to the maternally unmethylated ICR1 copy and thereby forms a chromatin boundary that blocks *IGF2* and promotes *H19* transcription of the maternal 11p15 genomic region. In contrast, the paternal methylation pattern forms alternative bounds and therefore expresses *IGF2*, which is involved in fetal development and growth.<sup>17</sup> While the contribution of *IGF2* to the pathoetiology of SRS has recently been shown,<sup>18</sup> the function of *H19* remains unknown, although it encodes several noncoding RNAs and binds factors that are implicates in epigenetic marks maintenance.<sup>19–21</sup>

The centromeric ICR2 in 11p15 regulates the expression of *CDKN1C*, *KCNQ1* (potassium channel KQT-family member 1) and further genes and is methylated only on the maternal allele. Loss-of-function mutations in the maternally expressed *CDKN1C* gene account for 5% of sporadic and up to 50% of familial BWS cases (Table 1) (for mutations see: <http://databases.lovd.nl/shared/genes/CDKN1C>). Gain-of-function mutations are associated with growth retardation syndromes (IMAGe syndrome, SRS). The gene encodes a cyclin-dependent kinase inhibitor (p57KIP2) and is part of the p21CIP2Cdk inhibitor family. Another noncoding RNA gene in 11p15, *KCNQ1OT1* (also known as LIT1), starts in intron 10 of the *KCNQ1* gene. *KCNQ1OT1* is expressed by the paternal allele and probably represses the expression of the *CDKN1C* gene on the same allele. Loss of methylation of the maternal ICR2 allele correlates with an increased expression of *KCNQ1OT1*. In BWS, one central physiological change caused by ICR2 (epi)mutations (hypomethylation at ICR2 as well as *CDKN1C* point mutations) is the reduced expression of (functional) *CDKN1C*.

## MOLECULAR DISTURBANCES IN SRS AND BWS

The types of mutations and epimutations in SRS and BWS affect the same genomic regions and genes, but in an opposite manner (Table 1). Four different types of molecular changes have been reported: epimutations (i.e. aberrant methylation at a DMR without a genomic alteration in the DMR itself), UPD, deletions/duplications of the DMRs and genomic point mutations in a gene the expression of which is regulated by the two germline DMRs. In particular, the identification of microduplications and microdeletions within 11p15.5 helped to define regulatory elements (i.e. DMRs, OCT4/SOX2 binding sites, enhancer motifs) responsible for a fine-tuned methylation and/or expression of 11p15.5 factors.<sup>22,23</sup> For molecular diagnostic testing, it is important to keep in mind that the predominant alterations in both

disorders, the *H19/IGF2:IG-DMR* hypomethylation in SRS and the upd(11)pat and 11p15 epimutations in BWS, can occur in a mosaic state.

## Silver–Russell syndrome

The majority of patients with the classical SRS phenotype<sup>7</sup> carry molecular changes in 11p15: hypomethylation of the *H19/IGF2:IG-DMR* accounts for 40–60% of patients.<sup>7</sup> About 1% of patients carry duplications or deletions affecting the region 11p15.5 (Table 1): the phenotype of these carriers is influenced by the size and gene content of the aberrations (for a review refs. 24–26). Upd (11p)mat and point mutations in the 11p15.5-encoded genes *CDKN1C* and *IGF2* have only been described once each.<sup>18,27,28</sup>

Between 4 and 10% of SRS patients carry maternal UPD of chromosome 7 (upd(7)mat) or segmental upd(7q)mat (for a review see Eggermann *et al*<sup>29</sup>), in single cases chromosomal imbalances affecting either the *GRB10* gene in 7p13 or the *MEST* gene in 7q32 have been reported (an LOVD entry for SRS-specific structural variations is currently in the submission process). Additionally, in a considerable number of patients (submicroscopic) alterations of chromosomes other than 7 and 11 may be detected.<sup>7,30,31</sup> A number of SRS patients exhibiting (epi)mutations in 14q32 has recently been reported, and these molecular alterations correspond to findings in patients with Temple syndrome (TS14, OMIM616222). TS14 is an ID with changes affecting the IG-DMR and/or *MEG3-DMR* in 14q32, and its phenotype<sup>32</sup> overlaps with SRS (for a review see Kagami *et al*<sup>33</sup>). In single cases, maternal uniparental disomy of chromosomes 16 and 20 (upd(16)mat, upd(20)mat) have been reported<sup>7,34</sup> (for a review see Eggermann *et al*<sup>35</sup>).

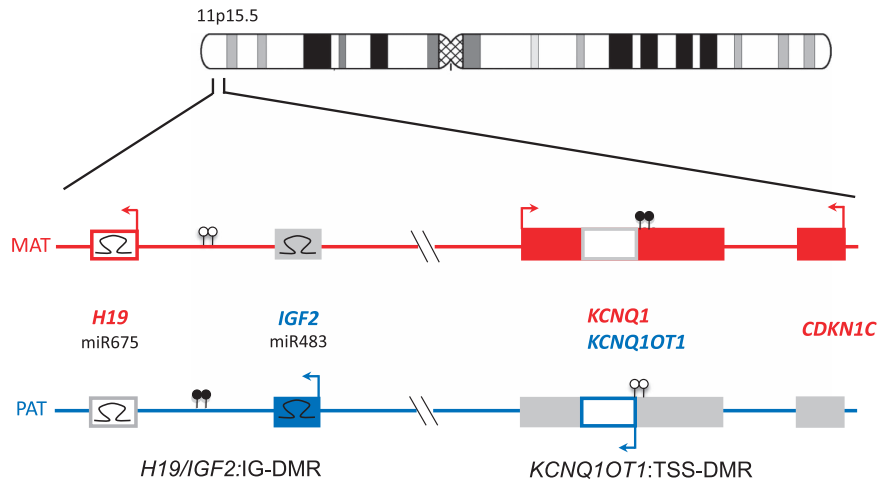
With the exception of patients with *H19/IGF2:IG-DMR* hypomethylation, the clinical findings in carriers of the other molecular changes do not always fit the clinical scoring suggested by different groups (for a review see Azzi *et al*<sup>7</sup>). In particular, in neonates and adults the decision on molecular testing cannot always be based on a convincing phenotype, but testing might also be applied to patients with less obvious growth parameters and dysmorphic signs.

## Beckwith–Wiedemann syndrome

In more than 80% of BWS patients chromosome 11p15.5 epimutations or mutations can be detected affecting the ICR1 and/or ICR2 DMRs (for a review see Brioude *et al*<sup>10</sup> and Mussa *et al*<sup>16</sup>) (Table 1). Most BWS cases are sporadic but familial inheritance is observed in up to 15% of all cases.<sup>11</sup>

The most frequent change is the hypomethylation of the *KCNQ1OT1:TSS-DMR*, accounting for up to 60% of patients. The second most frequent alteration in BWS is upd(11)pat, detectable in nearly 20%. The *H19/IGF2:IG-DMR* is affected by hypermethylation in 5–10% of BWS cases. For this subgroup, it has recently been shown that OCT4/SOX2 binding site mutations or deletions encompassing these binding sites within the ICR1 cause the aberrant methylation<sup>36</sup> by preventing *de novo* methylation of the maternal allele. Deletions, duplications and even balanced translocations in 11p15.5<sup>37,38</sup> also contribute to the mutational spectrum, with the size and parental origin of the affected region influencing the phenotype. *CDKN1C* mutations are not only frequent in familial cases but are also of importance in sporadic BWS with a frequency of 5%.<sup>39</sup> In the latter group, a significant number of patients exhibit a cleft palate.<sup>40</sup> Familial cases mainly present with *CDKN1C* mutations (50%), chromosomal duplications/deletions or mutations in other genes/regulative elements (eg, OCT4/SOX2, CTCF binding sites).





**Figure 1** The imprinted gene cluster in 11p15.5. It is divided in two functional domains whose imprinting is dependent on distinct ICRs (*H19/IGF2:IG-DMR*, *KCNQ1OT1:TSS-DMR*). (Filled boxes, protein coding genes; empty boxes, noncoding genes;  $\Omega$  miRNAs; filled lollipops, methylated regions; empty lollipops, unmethylated regions; red, genes expressed from the maternal (mat) chromosome; blue, genes expressed from the paternal (pat) chromosome. Arrows above the genes, transcription direction of sense genes; arrows below the genes, transcription direction of anti-sense genes)

### Multilocus imprinting disturbances

The association between aberrations at specific imprinted genes and distinct congenital disorders is well known, but the number of reports on patients with generally disturbed methylation patterns (MLID) has increased recently (for a review see Mackay *et al*<sup>41</sup>). While the patients often exhibit a specific ID phenotype, for example, BWS, molecular testing reveals that aberrant methylation does not only affect the disease-specific imprinted loci (eg, 11p15 in BWS) but also other imprinted regions. In BWS, MLID is detected in ~25% of patients with hypomethylation of the *KCNQ1OT1:TSS-DMRs*, and in SRS in 7–10% of *H19/IGF2:IG-DMR* patients (Table 1). A common cause of MLID has not yet been reported, but mutations in *trans*-acting genes/factors have been identified in some cases.<sup>42–44</sup> Thus, whole-exome/genome sequencing strategies or candidate gene analyses might be considered in these patients on a research basis and in cooperation with reference centers. Assisted reproductive technologies have also been associated with an increased risk of MLID.<sup>45</sup>

### MOLECULAR GENETIC TESTING STRATEGIES

Several diagnostic assays for the molecular diagnosis of SRS and BWS have been developed (Table 2), but the decision on the tests to be applied in a laboratory depends on many factors, including the equipment of the laboratory, the methodological experience, the pattern of referral, the in-house guidelines for diagnostic workup and the national strategy of diagnostic workup.

In any case, the laboratory has to be aware of the advantages and limitations of a test, and the diagnostic workup should follow the diagnostic algorithms shown in Figure 2. This diagnostic algorithm refers to samples received to confirm the clinical diagnosis of SRS or BWS. It does not account for cases referred for UPD analyses based on chromosomal findings or a precedent (family) history with an already known molecular disturbance.

#### First step: Methylation test for the 11p15.5 DMRs in SRS and BWS and for chromosome 7 in SRS

In both SRS and BWS, a methylation-sensitive (methylation-specific (MS)) approach targeting the two DMRs in 11p15.5 is recommended (Figure 2). In case of SRS, first-step analysis also has to include chromosome 7 methylation testing. Indeed, the majority of patients

with chromosome 7 alterations carry an UPD of the whole chromosome, but there is a growing number on reports of patients with a segmental UPD for 7q (upd(7q)mat) including *MEST*.<sup>29</sup> Furthermore, several patients with copy number variations affecting either the *GRB10* locus in 7p13 or the *MEST* imprinted region in 7q32 have been reported, thus both imprinted regions have to be analyzed.

#### Further procedure for SRS and BWS after a positive testing result

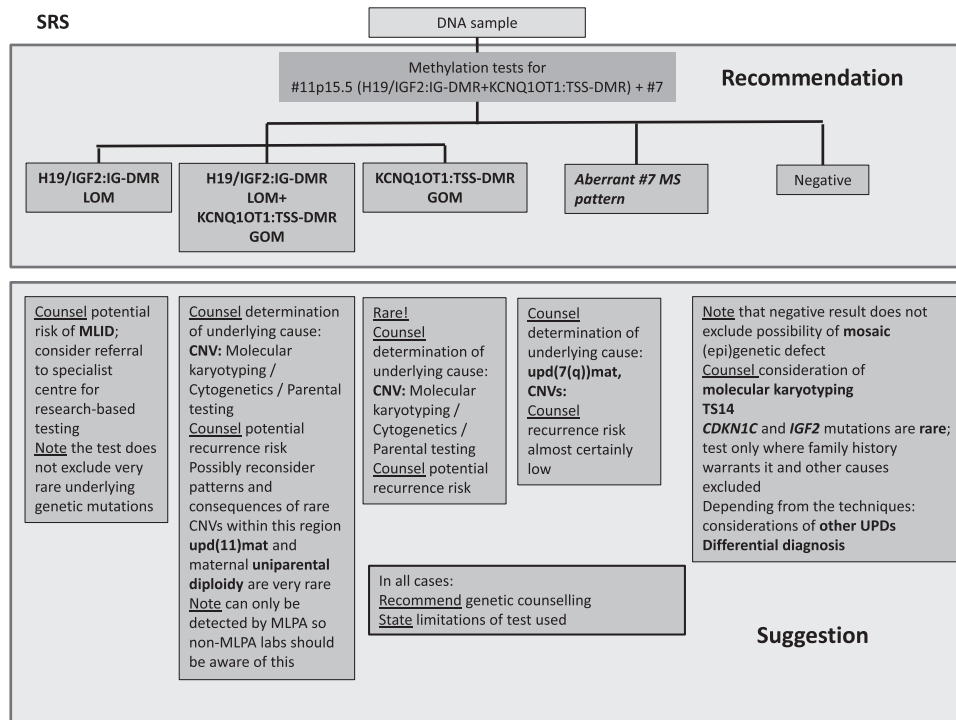
With a positive result of methylation testing for 11p15.5 (or chromosome 7), the clinical diagnosis is confirmed. However, the discrimination between the different molecular subtypes is required (epimutations, UPDs, copy number variations (CNVs), translocations) as the basis for a personalized clinical management in BWS, and a directed genetic counseling in both disorders.<sup>16</sup> Depending on the applied method, this discrimination may be possible in a single analysis or multiple tests have to be carried out (Figure 2). It depends on the diagnostic repertoire of the lab whether it offers this workup or whether it forwards the samples to reference centers. In case only the first step of diagnosis is performed, the required subsequent diagnostic steps and putative molecular results must be included in the report, including the associated recurrence risks (Figure 2 and Table 1).

#### Further procedure for SRS after a negative testing result

In case of a negative 11p15.5 and chromosome 7 testing result, testing for TS14-associated alterations and molecular karyotyping can be considered (Figure 2).

In contrast, mutations in *CDKN1C* and *IGF2* have only been described in a single family each,<sup>18,27</sup> and screening studies in larger cohorts of sporadic SRS patients have so far been negative, and therefore these genes should only be analyzed after a careful re-evaluation of clinical findings and family history. The sequencing should not be included in routine diagnostic analysis for SRS. Additionally, differential diagnoses have to be considered (for a review see Saal<sup>1</sup>).

Indeed, further molecular changes have been identified in single cases (eg, upd(16)mat, upd(20)mat); thus, if warranted by the clinical features and/or family history of the patient, testing for these very rare (epi)mutations may be considered.



**Figure 2** Diagnostic algorithm for SRS (a) and BWS (b). For both disorders, methylation-specific tests for both imprinted regions in 11p15.5, and in case of SRS for the DMRs on chromosome 7 are recommended. The lab has to be aware of the possible outcomes and their interpretation. In case of a positive testing result, the subsequent discrimination is mandatory and has at least to be suggested in the report. Ditto, the further proceeding after a negative result has to be advised by the reporting geneticists.

The complex molecular workup in SRS, including differential diagnosis (see below) can, in part, be circumvented by replacing single-step analyses for each imprinted locus by tests targeting different DMRs simultaneously in the same run ('multilocus testing'), thereby allowing the one-step identification of aberrant methylation patterns at different DMRs caused by other imprinting defects (eg, TS14, MLID) or uniparental diploidy.

#### Further procedure for BWS after a negative testing result

In case of a negative result of the methylation test for the 11p15.5 DMRs in a BWS patient, sequencing of the coding exons and the exon–intron boundaries of the *CDKN1C* gene should be considered in case of a positive family history, cleft palate and abdominal wall defect (umbilical hernia or exomphalos).

Nevertheless, nearly 30% of patients with a characteristic BWS phenotype still remain without a molecular diagnosis, and differential diagnoses should also be discussed after clinical re-evaluation (for a review see Shuman *et al*<sup>11</sup>).

#### Mosaicism in SRS and BWS

Mosaicism affects the majority of molecular disturbances in SRS and BWS,<sup>7,41</sup> that is, in a given sample the (epi)genetic defect is present only in a fraction of the total number of cells. Low-level mosaicism within a tissue may not be detected because of the detection limit of the methods used, leading to false-negative results. The fraction of aberrant cells may vary among different tissues. After clinical re-evaluation testing other tissues might be considered to exclude tissue-specific mosaicism.

#### MOLECULAR GENETIC TESTING METHODS

Several techniques have been implemented in the molecular diagnostic testing algorithm of both SRS and BWS, and as the first EMQN pilot schemes for SRS and BWS in 2014 showed (<http://www.imprinting-disorders.eu/wp-content/uploads/2015/07/EMQN-SRS-Summary-Report.pdf>; <http://www.imprinting-disorders.eu/wp-content/uploads/2015/07/EMQN-BWS-Summary-Report.pdf>), different methods (Table 2) are suitable for diagnostic analysis. However, the choice of a technique to identify 11p15.5 and chromosome 7 disturbances should consider the loci to be analyzed and their precise genomic localization, the investigated tissue and the spectrum of occurring mutations and epimutations. Furthermore, none of these tests are certified for diagnostic use. Therefore, they have to be fully validated in the laboratory, and the normal reference ranges may be challenged by the possible occurrence of mosaicism. Furthermore, MS-specific tests should contain controls for complete digestion or bisulfite treatment.

Several methods do not allow the discrimination between the different types of molecular changes (upd, CNVs, epimutations) (Table 2), in this situation a combination of techniques has to be applied.

#### MS-specific techniques

Assays targeting altered methylation patterns can be roughly subdivided into two groups, those based on bisulfite-converted DNA and those using methylation-sensitive restriction enzymes (Table 2). With the exception of Southern blotting, all MS assays are based on the hybridization of short probes or primers; therefore, these tests are prone to be affected by single-nucleotide polymorphisms (SNPs) influencing the hybridization efficacy of complementary probe–primer sequences. Thus, false-positive results might occur, and positive results

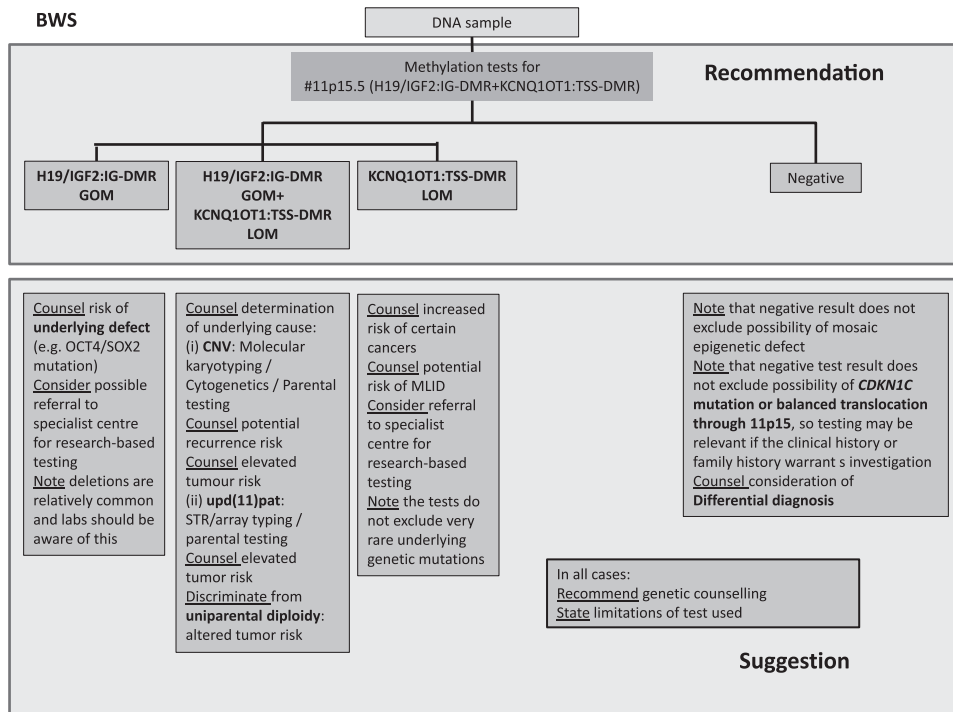


Figure 2 Continued.

should therefore be confirmed – if available – by a second technique or other probes–primers, or at least by a second independent run.

The EMQN pilot scheme revealed that MS-specific multiplex ligation probe-dependent amplification (MLPA) is by far the most widely used test (>80%) in routine diagnostics for SRS and BWS and will be described below in more detail; for the other tests see Table 2.

MS-MLPA (Supplementary Figure 1) as an adaption to normal MLPA provides a tool to simultaneously detect changes of copy numbers and methylation. DNA is digested with a methylation-sensitive restriction enzyme after hybridization of the MLPA probes. Comparison of peak heights of undigested and digested DNA indicates the methylation level of a particular probe. Monitoring complete digestion is an essential quality control of the experiment. MS-MLPA just requires general laboratory equipment (thermocycler, capillary electrophoresis platform), and the bioinformatics analysis can be performed by freely available or commercially available software packages (eg, Coffalyser.Net (JSI, Freiburg, Germany); GeneMarker software (SoftGenetics, State College, PA, USA)). For data normalization, the parallel analysis of samples with normal genotype/epigenotype is recommended by the manufacturer. Quality and purity of DNA is an essential prerequisite for definitive MLPA data interpretation, and the labs should therefore be aware of it. In principle, DNA samples isolated with different methods and from different tissues might be used in parallel in the same experiment, but the methylation pattern in the tissues tested must be known and identical to the tissue tested. According to general good practice laboratory guidelines, a non-template control and at least one positive control should be run in parallel. Several MS-MLPA Kits are commercially available from MRC Holland (Amsterdam, Netherlands; <http://www.mrc-holland.com>).

*Chromosome 11p15.5 testing (ME030 BWS/RSS Kit, MRC Holland).* The assay includes more than 20 probes specific for the region

11p15.5. Two probes serve as a control of the *HhaI* digestion in the methylation run. As controls for copy number quantification, probes outside 11p15.5 are included. Ten of the 11p15.5-specific probes are methylation-specific and target the germline *H19/IGF2:IG-DMR* (four probes) and *KCNQ1OT1:TSS-DMR* (four probes), additionally one probe each for the somatic DMRs of *IGF2* and *CDKN1C* is included. The analysis of the same germline DMR by several probes reduces the risk of false-positive or false-negative results due to SNPs. Furthermore, if one probe fails and artificial hypomethylation of single probes occurs (eg, due to DNA extraction method or cell culture), there are three remaining probes to assess the methylation status.

The dosage analysis of the kit allows the identification of duplications and deletions in 11p15.5. In cases of a large duplication affecting the whole 11p15.5 region all probes are affected. Additionally, it also detects smaller CNVs restricted to parts of the region. However, the precise size and content of 11p15.5 CNVs should be determined by further suitable methods (eg, molecular karyotyping, qPCR).

Owing to the opposite imprinting patterns of the two 11p15.5 DMRs (*H19/IGF2:IG-DMR* is paternally methylated and *KCNQ1OT1:TSS-DMR* is maternally methylated), the methylation analysis of the ME030 assay allows both the identification and discrimination between the epimutations, and, in combination with the dosage analysis, between epimutations, UPDs and CNVs affecting both DMRs. Indeed, segmental UPDs of only one of the DMRs might be indistinguishable from isolated epimutations, but these have not yet been reported. In these situations, microsatellite typing might be applied.

*Chromosome 7 testing (ME032 UPD7-UPD14 Kit, MRC Holland).* With this assay, upd(7)mat can be identified (Supplementary Figure 1). Similar considerations as for the interpretation of the ME030 Kit should be made, but in contrast to the 11p15.5 assay, and because of the methylation status of the targeted DMRs, a



differentiation between UPD and epimutations is not possible and requires additional workup. However, with the exception of isolated hypermethylation of the *GRB10* DMR,<sup>46</sup> epimutations have not yet been reported. The advantage of this assay is the inclusion of imprinted loci on chromosomes 14 and 6, and thus molecular changes associated with TS14 as well as some MLID patterns can be detected in parallel.

#### Non-MS assays

Depending on the design and informativity of the initially applied MS test, an identification, confirmation and further characterization of genomic alterations (CNVs, SNPs, UPD) might be required. For these purposes, the following assays have been implemented with different advantages and limitations (Table 2):

*Microsatellite typing* (short tandem repeat typing, STR) is frequently applied for detection of UPDs, in particular in prenatal testing. In addition to the patient's DNA sample, a sample from at least one parent is required to delineate the parental origin of the two STR alleles. For whole arm UPD detection STRs are an efficient and inexpensive tool, but it has to be considered that a discrimination between (mosaic)UPD and deletions or duplications are not possible in every case (Supplementary figure 2). Furthermore, segmental UPDs of small fragments might escape detection. In case UPD has been determined solely by STR typing, the lab should be aware of the scenario of non-paternity. Microsatellite markers suitable for UPD analysis in context with SRS and BWS should be selected on the basis of their informativity (PIC > 0.7) and their genomic localization (markers used in diagnostic testing are listed in Supplementary table 2).

*Molecular karyotyping* (Chromosomal Microarray) is a suitable method to determine the size and gene content of CNVs, but it has to be considered that the probe content of arrays is not standardized, and different assays have different coverages of the regions of interest. Therefore, very small imbalances might remain undetected and might rather be analyzed by qPCR. By SNP array analysis, uniparental isodisomic segments can in principal be detected and are therefore applicable in BWS testing,<sup>47</sup> but small isodisomic segments cannot be discriminated from small homozygous stretches, and heterodisomy can only be identified by parallel analysis of parental samples and the use of additional bioinformatic tools. aCGH is not suitable for UPD detection.

Identification of single-nucleotide variants/point mutations should be performed by genomic *sequencing*, analyzing the genomic sequences defined as reference sequences (Supplementary Table 2). In case of sequencing genes (eg, *CDKN1C*), the appropriate guidelines (eg, of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (2015)<sup>48</sup> or the Association for Clinical Genetic Sciences<sup>49</sup> ([http://www.acgs.uk.com/media/774853/evaluation\\_and\\_reporting\\_of\\_sequence\\_variants\\_bpgs\\_june\\_2013\\_-\\_final.pdf](http://www.acgs.uk.com/media/774853/evaluation_and_reporting_of_sequence_variants_bpgs_june_2013_-_final.pdf))) should be followed. It must be noted that sequencing analysis of *CDKN1C* is complex as the gene is GC-rich and contains several non-pathogenic in-frame deletion variants.

#### Future methodological developments

Owing to the heterogeneous molecular findings in SRS and BWS, and the limitations of several of the currently applied tests, the diagnostic workup including discrimination of the molecular subtypes might become time-consuming and expensive. Therefore, the development of multilocus tests is in progress, either on the basis of conventional MS-MLPA by combining probes from different imprinted loci or by next-generation sequencing approaches. The latter harbors the

potential to be more sensitive, as massive parallel sequencing might allow the detection of low-level mosaicism, and comprehensive inclusion of possible genomic and epigenetic changes.

#### INTERPRETATION OF DIAGNOSTIC TESTING RESULTS

The precise identification and discrimination of the mutations or epimutations in patients referred with SRS or BWS features is the prerequisite for clinical management in BWS and a well-directed genetic counseling.

#### LOM at one of the 11p15.5 DMRs in SRS and BWS/MLID

LOM (Hypomethylation) at one of the two DMRs in 11p15.5 represents the most frequent diagnostic finding in the two 11p15.5-associated IDs (Table 1) and their identification confirms the clinical diagnosis of SRS and BWS, respectively. In general, the recurrence risk is low in these molecular subgroups,

However, there are reports on families with two BWS sibs,<sup>43,50</sup> with one sib showing a *KCNQ1OT1*:TSS-DMR hypomethylation and the second one showing the same epimutation but as part of an MLID. Although this situation seems to be rare, it illustrates that MLID also has to be considered in BWS testing. In general, MLID accounts for 7–10% of SRS with *H19/IGF2*:IG-DMR hypomethylation, and for 25% of patients with *KCNQ1OT1*:TSS-DMR hypomethylation in BWS. Assuming a general frequency for this molecular subtype of 50% in BWS cohorts, MLID is detectable in 12.5% of BWS patients. In case of MLID the recurrence risk is difficult to estimate because mutations in genes associated with a generally disturbed methylation have only been recently reported. The disease-causing variants may either have an autosomal recessive effect (eg, *ZFP57* in transient neonatal diabetes mellitus<sup>42</sup>) or represent the recently defined maternal effect mutations, that is, the maternal genotype is responsible for the aberrant methylation in her offspring.<sup>43,44</sup>

#### GOM at one of the DMRs in 11p15.5

Up to now, isolated GOM (hypermethylation) in 11p15.5 has been reported for BWS: hypermethylation of the *H19/IGF2*:IG-DMR might be difficult to detect in case of a low-level mosaicism. Although GOM mainly occurs sporadically, up to 20% of GOM patients carry genomic disease-causing variants within the *OCT4/SOX2* binding site (BS) 5' to the DMR<sup>36</sup> and referral to specialist research centers can be considered. In case of detection of an *OCT4/SOX2* BS pathogenic variants, it has to be evaluated individually. Another group of genomic variants affecting the *H19/IGF2*:IG-DMR are microdeletions that reduce the number of CTCF target sites and might be associated with the inactivation of the DMR: their size and spacing influence the degree of DNA methylation and the clinical phenotype.<sup>22</sup> Careful characterization of these microdeletions is therefore needed to predict recurrence risks and phenotypical outcomes. In these cases, analysis of parental DNA should be considered to determine the mode of transmission, and the recurrence risk is 50% in the event of a maternally inherited disease-causing variant.

In SRS, GOM of the *KCNQ1OT1*:TSS-DMR is extremely rare and can be due to a small duplication of the maternal allele, thus requiring a careful workup by a specialized lab.

#### Detection of both LOM/GOM at the two 11p15.5 DMRs

Parallel occurrence of both LOM and GOM at the two 11p15.5 DMRs in the same patient either indicates UPD or CNVs. To discriminate these two alterations copy number analysis is required.

In BWS *upd(11)pat* contributes to up to 20% of cases, and it is mainly segmental (ie, restricted to the 11p15.5 region or the short arm

of chromosome 11, and isodisomic),<sup>47</sup> but some cases with upd(11)pat affecting the whole chromosome have been reported. STR determination of uniparental isodisomy can be hampered by the mosaic presence of the disturbance. Therefore, it might not only become a qualitative analysis but also a quantitative one. For upd(11)pat a postzygotic origin has been delineated and the recurrence risk is therefore not increased. Particular attention should be directed to (mosaic) uniparental diploidy (for a review see Kalish *et al*<sup>51</sup>). This genetic constitution is associated with further features additional to those of BWS, in particular with a severely increased risk for a broad-spectrum of (embryonal) tumors, and clinical surveillance and management is not covered by the conventional BWS tumor programs. A further problem in upd(11)pat is the variable degree of mosaicism, making the identification difficult or leading to a false interpretation: the increase of hybridization of the *H19/IGF2:IG-DMR* probes might be weaker than the decrease of the *KCNQ1OT1:TSS-DMR* probes, and might escape the detection.<sup>47</sup> The application of a second method might be helpful to confirm the molecular suspicion of UPD, even in case the *H19/IGF2:IG-DMR* is in the upper control range.

*Upd(11)mat* is extremely rare in SRS,<sup>28</sup> and only one case with mosaic maternal uniparental diploidy has been reported.

(Submicroscopic) *chromosomal imbalances in 11p15.5* (CNV), that is, duplications, confirm the clinical diagnosis of SRS or BWS. After identification of a CNV in an SRS or a BWS patient, molecular karyotyping is indicated to confirm the abnormality and to determine its size as the content of genes and regulative elements of the aberration might explain additional clinical features of the patient, which might not belong to the SRS/BWS spectrum (an LOVD entry for SRS-specific CNVs is currently in the submission process). A cytogenetic/molecular-cytogenetic analysis of the patients and their parents might help to determine the nature of the imbalance and to estimate the recurrence risk in case of a familial rearrangement.

In case of a familial rearrangement, an autosomal-dominant inheritance with phenotypic expression depending on the sex of the contributing parent can be expected: in case the duplication affects the whole 11p15.5 region of the paternal allele, a BWS phenotype is probable, whereas an SRS-like clinical picture will occur in case the maternal chromosome carries the duplication. Thus, both phenotypes may occur in the same family.<sup>26</sup>

#### Smaller CNVs affecting only one of the 11p15.5 DMRs

Smaller duplications or deletions affecting only parts of the two 11p15.5 imprinting regions, may cause BWS, SRS or a normal phenotype, depending on the regulative and coding DNA sequences involved in the rearrangement and the sex of the contributing parent (for a review see Begemann *et al*<sup>25</sup>). Thus, a careful workup has to be applied in case of duplications or deletions of the imprinted regions in 11p15.5, and the geneticist has to estimate recurrence risks on the basis of the up-to-date literature.

#### Upd(7)mat in SRS patients

As the vast majority of upd(7)mat are products of a trisomy rescue, the recurrence risk is generally low and the only predisposing factor is advanced maternal age. However, two cases have been reported in which the upd(7)mat was the result of a familial chromosomal translocation, in this situation the recurrence risk is increased.<sup>52,53</sup> Thus, cytogenetic/molecular-cytogenetic analysis should be considered whenever there is a chance for a chromosomal aberration (eg, if a translocation or aberration in the family involving chromosome 7 or 11 is known), but need not be generally offered to UPD cases.

#### Pathogenic variants (in *CDKN1C* and *IGF2*)

In BWS, *CDKN1C* mutation analysis has to be considered owing to its frequency of up to 50% in familial cases. Indeed, in some labs it is performed as the first diagnostic step in families with BWS and in BWS patients with cleft palate. If a pathogenic variant in one of the genes is identified, genetic counseling should be offered and a segregation analysis in the families can be discussed. Comparable to chromosomal rearrangements, the recurrence risk is 50% for *CDKN1C* and *IGF2* mutations, but it depends on the sex of the transmitting parent. A BWS phenotype will occur if a *CDKN1C* disease-causing variant is inherited from the mother, or if a *de novo* mutation affects the maternal allele, whereas there is no (or minimal) phenotypic effect in case the paternal gene copy carries the pathogenic variant. For SRS, maternal inheritance of a missense mutation located in the PCNA-binding domain of *CDKN1C* may lead to an SRS or IMAGe phenotype, whereas paternal inheritance of an *IGF2* mutation results in SRS.

#### Normal chromosomes 11p15.5 and 7 methylation results in SRS

Normal chromosomes 11p15.5 and 7 methylation patterns do not rule out the clinical diagnosis of SRS, currently 30–40% of patients remain without a molecular confirmation of the clinical diagnosis.<sup>7</sup> In routine screening without strict diagnostic inclusion criteria the detection rate is even lower.<sup>8</sup> As mentioned before, molecular changes in the imprinted 14q32 region significantly contribute to the mutation spectrum<sup>7,8,33</sup> and therefore overlap with TS14. TS14 testing is thus suggested as a further diagnostic step for patients with strong clinical suspicion of SRS, and it is already included in a commercially available MS-MLPA assay. Similar to that for SRS and BWS, TS14 testing should consist of appropriate assays to discriminate between the different molecular subgroups.

Furthermore, molecular karyotyping is indicated as a further molecular diagnostic step in patients with clear phenotypic features, and significantly contributes to the clarification of the cause of the phenotype and recurrence risks.

Owing to the mosaic tissue distribution of 11p15.5 epimutations,<sup>2</sup> testing of other tissues (eg, fibroblasts) might also be considered after a careful clinical re-evaluation.

#### Normal 11p15.5 methylation result in BWS

The aforementioned (epi)mutations in the two germline DMRs in 11p15.5 account for 70–80% of patients with BWS, thus a negative result does not rule out BWS (Table 1). However, epimutations and upd(11)pat might escape detection owing to uneven mosaic distribution.<sup>54</sup> Thus, the analysis of another tissue for epimutation might be considered if the routine 11p15.5 testing is negative.

For BWS no major further molecular subgroups have been identified, but differential diagnosis should also be considered.

#### REPORTING

For different reasons it is not recommended to use the same templates for sample referral, reporting and interpretation of findings for BWS and SRS.

In general, the format of the reports has to correspond to general guidelines of reporting molecular genetic results according to International and the National Regulators (eg, from the Association for Clinical Genetic Science (Part of the British Society for Genetic Medicine; [http://www.acgs.uk.com/media/949852/acgs\\_general\\_genetic\\_laboratory\\_reporting\\_recommendations\\_2015.pdf](http://www.acgs.uk.com/media/949852/acgs_general_genetic_laboratory_reporting_recommendations_2015.pdf)). Laboratories reporting (BWS/SRS) testing results should participate annually at least in external quality assessment. It is recommended that testing laboratories

are accredited to international standards, for example, ISO 15189 or equivalent. In particular, it is essential that the report contains the following items:

- unequivocal patient identifiers, numbering of pages,
- the reason for referral,
- source of DNA sample (eg, blood, buccal smear, amniocytes),
- a consistent naming of the analyzed DMRs should be used in the same report, and recently a common nomenclature has been suggested the European Network of Congenital Imprinting Disorders EUCID.net ([www.imprinting-disorders.eu](http://www.imprinting-disorders.eu)) (Supplementary Table 1). The nomenclature of genomic mutations has to be in accordance with the suggestions of the HGVS (<http://www.hgvs.org/mutnomen/>),
- applied methods and statement on the limitations and sensitivities including detection of mosaicism. Appropriate references should be given, if commercial kits are used, the kit version has to be mentioned (eg, ME030-C3; <http://www.mrc-holland.com>),
- the precise result description,
- interpretation of diagnostic testing results (see above),
- statement whether the clinical diagnosis is confirmed or not,
- statement on the significance of the molecular results for the clinical management: for SRS, the certainty of the molecular genetic diagnosis aids medical management and precludes further investigations for short stature. However, therapeutic measures such as growth hormone treatment, physiotherapy or managing of hypoglycemic are based on clinical symptoms rather than on the genetic diagnosis, and should therefore not be suggested in the report. In case of BWS, tumor surveillance should be advised in the report according to the national guidelines and as suggested by the Clinical Utility Gene card for BWS.<sup>9</sup>
- in case the results of only single steps of the diagnostic algorithm (Figure 2) are reported, the consecutive steps must be suggested,
- genetic counseling has to be recommended, and recurrence risk has to be mentioned.

## PRENATAL TESTING

The growing knowledge on the molecular basis of SRS and BWS and the increasing number of positively tested patients results in an increasing demand for prenatal testing for these diseases. Owing to the complexity of molecular testing and the numerous questions that raised the context of prenatal testing, and ranging from methodological questions to ethical topics, the authors have addressed this topic separately.<sup>55,56</sup> In principal, prenatal testing might be offered in specific situations, but the decision on prenatal testing for SRS or BWS requires careful and comprehensive discussions (including the limitations of methods and information value, ethical issues) between the families, the genetic counselors, the obstetrics and laboratories.

## DIFFERENTIAL DIAGNOSIS

In both 11p15.5-associated IDs, the clinical diagnosis is often difficult because of its variable presentation during childhood and adolescence (eg, with age the facial characteristics tend to become attenuated in SRS, and normalized in BWS); furthermore, the molecular and phenotypic findings overlap with other IDs and syndromes. Thus, after exclusion of the major molecular changes detectable in SRS or BWS (Figure 2), a clinical re-evaluation should be performed and differential clinical diagnoses should be considered (eg, IMAGE syndrome, 3M syndrome for SRS; Sotos, Weaver, Perlman, Simpson–Golabi–Behmel syndrome for BWS).<sup>1,11</sup> Recent reports indicate

the contribution of new, so far neglected loci and chromosomes to SRS: patients with upd(16)mat or upd(20)mat show symptoms overlapping with SRS.<sup>7,34</sup> However, it is still too early to include them in routine diagnostic workups, and they should rather be included in research strategies.

## CONCLUDING REMARKS

These guidelines are based on the knowledge acquired from peer-reviewed and published data, as well as observations of the authors in their practice. Current practice of molecular testing and reporting of SRS and BWS was assessed by a first external pilot quality assessment scheme organized by the EMQN in 2014. In the two-pilot schemes for SRS and BWS, a total of 42 laboratories from 19 European and non-European countries for BWS and 31 diagnostic centers from 14 countries for SRS participated. Based on the results from these schemes and in context with activities of the European Network of Imprinting Disorders (EUCID.net) towards a consensus in diagnostics and management of SRS and BWS, best practice guidelines have been developed. Members of institutions working in the field of SRS and BWS diagnostics were invited to comment, and in the light of feedback amendments were made. The final document was ratified in the course of an EMQN best practice guideline meeting for 11p15.5 IDs on 3 October 2015 and in accordance with the general SRS and BWS consensus guidelines, which are in preparation.

These guidelines can only provide a snapshot of current knowledge at the time of manuscript submission. Readers are advised to keep up with the literature.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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