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# Molecular testing for imprinting disorders

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**Abstract:** Imprinting disorders are a group of rare diseases with a broad phenotypic spectrum caused by a wide variety of genetic and epigenetic disturbances of imprinted genes or gene clusters. The molecular genetic causes and their respective frequencies vary between the different imprinting disorders so that each has its unique requirements for the diagnostic workflow, making it challenging. To add even more complexity to this field, new molecular genetic causes have been identified over time and new technologies have enhanced the detectability e. g. of mosaic disturbances.

The precise identification of the underlying molecular genetic cause is of utmost importance in regard to recurrence risk in the families, tumour risk, clinical management and conventional and in the future therapeutic managements.

Here we give an overview of the imprinting disorders, their specific requirements for the diagnostic workup and the most common techniques used and point out possible pitfalls.

**Keywords:** imprinting disorders, molecular genetic testing, copy number variant, single nucleotide variant, uniparental disomy, imprinting defect, MS-MLPA

## Introduction

Due to their molecular heterogeneity and the continuous reports on new molecular findings, diagnostic testing of imprinting disorders (ImpDis) is challenging and each entity shows a different pattern of molecular disturbances (Table 1). Therefore, diagnosis in this field requires an updated overview on the molecular disturbances detectable in a specific disorder, but also knowledge on the molecular and clinical overlap with other entities. In addition, the postzygotic origin of several molecular alterations results in a tissue-specific mosaic distribution which hampers detection and can cause false-negative results. In the case of copy number variations (CNVs) affecting imprinted regions, the extent and genomic content have to be considered.

In the majority of ImpDis, up to four different types of molecular alterations can be discriminated, including uniparental disomy (UPD), pathogenic CNVs and pathogenic single nucleotide variants (SNVs), as well as imprinting defects (for review, see Prawitt and Haaf in this issue). Whereas UPDs and pathogenic CNVs and SNVs represent genomic alterations, imprinting defects (also called epimutations) are defined as aberrant methylation marks at differentially methylated regions (DMRs). In the majority of imprinting defects, the molecular causes are currently unknown (so-called primary epimutations), but there is an increasing number of reports on genomic alterations with an impact on the establishment or maintenance of imprinting marks causing imprinting defects (secondary epimutations) [1]. The precise identification of the molecular correlate of an ImpDis is therefore not only relevant for conventional and – in the future – precise therapeutic managements, but also for genetic counselling (see Elbracht et al. and Horsthemke and Zechner in this issue).

## Genetic testing strategies and tests

A broad spectrum of diagnostic assays to identify disturbances affecting imprinted regions have been implemented (for review, see Ref. [2]), but the decision on testing strategies for a specific ImpDis should primarily be based on the spectrum and frequencies of the different molecular alterations (Table 1). Additionally, it might also be influenced by the methodological experience of the laboratory and in-house guidelines. In any case, the laboratory has to appreciate the advantages and limitations of a method, and recommended diagnostic algorithms should be followed if available (e. g. Refs. [3, 4, 5]; see also Figure 1).

In the more frequent ImpDis Prader–Willi syndrome (PWS), Angelman syndrome (AS), Silver–Russell syndrome (SRS) and Beckwith–Wiedemann syndrome (BWS), as well as in the chromosome 14-associated entities Temple syndrome (TS14) and Kagami–Ogata syndrome (KOS14), the three major molecular subtypes are

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**Table 1:** Overview on the molecular findings in the currently known ImpDis. For the clinical features, we refer to the contribution of Elbracht et al. in this issue (NR, not reported).

| Imprinting Disorder | OMIM# | Chromosome | UPD | Pathogenic CNV | Imprinting Defect | Mosaicism | Pathogenic SNVs*** | GeneReviews and/or guidelines | major reference (for frequency of molecular subgroups) |
|---|---|---|---|---|---|---|---|---|---|
| Transient Neonatal Diabetes mellitus (TNDM) | 601410 | 6q24 | upd(6q24)pat: 41 % | dup(6q)pat: 33 % | *PLAGL1*:alt-TSS-DMR, LOM: 26 %** | NR | in MLID: ZFP57 | yes | see Genereviews |
| Birk-Barel intellectual disability syndrome (BBIDS) | 612292 | 8q24 | NR | NR | NR | NR | KCNK9 (maternal allele) | – | [37] |
| Silver-Russell syndrome (SRS) | 180860 | 7<br>11p15.5 | upd(7)mat: 7–10 %****<br>upd(11p)mat: 1 case | dup(7p/q)mat: single cases<br>dup(11p)mat: <1 %**** | *GRB10*:alt-TSS-DMR, GOM: 1 case<br>*H19/IGF2*:TSS-DMR, LOM: 40 %, **/****<br>*KCNQ1OT1*:TSS-DMR, LOM: single cases* | NR<br>yes | NR<br>*CDKN1C, IGF2* | yes [28] | [28] |
| Beckwith-Wiedemann syndrome (BWS) | 130650 | 11p15.5 | upd(11p15)pat: 20 %**** | dup(11p)pat: <1 %**** | *H19/IGF2*:TSS-DMR, GOM: 4 %****<br>*H19/IGF2*:TSS-DMR, LOM: single cases*<br>*KCNQ1OT1*:TSS-DMR, LOM: 50 %**/**** | yes | *CDKN1C* (maternal allele): sporadic 5 %****, familial 25–50 % | yes [29] | [29] |
| Temple syndrome (TS14) | 616222 | 14q32 | upd(14)mat: ~70 % | del(14q32)pat: ~10 % | *MEG3*:TSS-DMR, LOM: ~20 % | yes | NR | | [23] |
| Kagami-Ogata syndrome (KOS14) | 608149 | 14q32 | upd(14)pat: ~70 % | del(14q32)mat: ~20 % | *MEG3:TSS*-DMR, GOM: 10 % | single cases | NR | | [21, 22] |
| (familial) Central Precocious Puberty (CPPB) | | 14q32 | NR | single cases | NR | NR | *DLK1* (paternal allele): loss-of-function | – | [27] |
| Prader-Willi syndrome (PWS) | 176270 | 15q11q13 | upd(15)mat: 25–30 % | del(15q11q13)pat: 70–75 % | *SNURF:TSS*-DMR, GOM: 1 % (in 10–15 % due to an IC deletion) | single cases | NR | yes [11] | [11] |
| Angelman syndrome (AS) | 105830 | 15q11q13 | upd(15)pat: 1–2 % | del(15q11q13)mat: 75 % | *SNURF:*TSS-DMR, LOM: 3 % (in 10–15 % due to an IC deletion) | yes | *UBE3A* (maternal allele): 10 % | yes [11] | [11] |
| Central Precocious Puberty 2 (CPPB2) | 615346 | 15q11.2 | NR | NR | NR | NR | *MKRN3* (paternal allele) | | [20] |

**Table 1:** (continued).

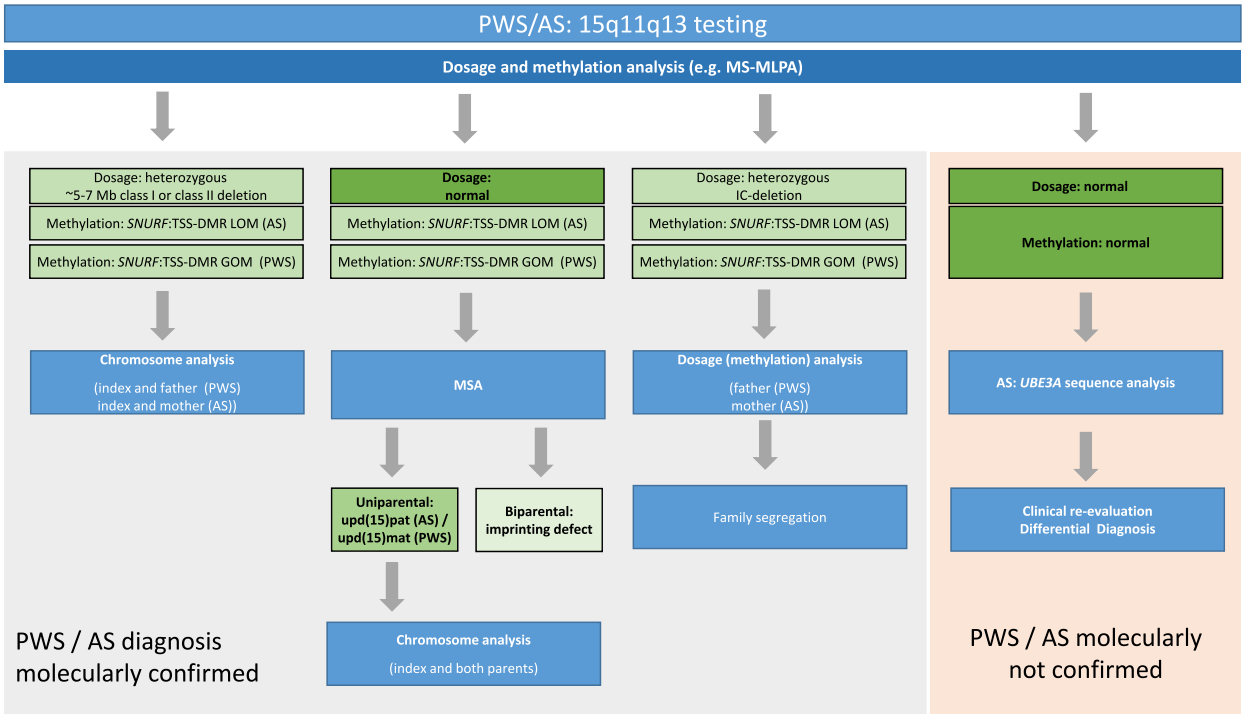
| Imprinting Disorder | OMIM# | Chromosome | UPD | Pathogenic CNV | Imprinting Defect | Mosaicism | Pathogenic SNVs*** | GeneReviews and/or guidelines | major reference (for frequency of molecular subgroups) |
|---|---|---|---|---|---|---|---|---|---|
| Schaaf-Yang syndrome (SHFYNG) | 615547 | 15q11.2 | no | no | no | NR | *MAGEL2* (paternal allele): truncating mutations only | | [18] |
| Pseudo-hypopara-thyoridism type 1B (PHP1B) | 603233 | 20q13 | upd(20)pat: 2–20 % | del(20q13)mat: single cases | *GNAS-NESP:TSS*-DMR, GOM/*GNAS-AS1:TSS*-DMR, LOM/*GNAS-XL:Ex1*-DMR, GOM: >60 %** | yes | *GNAS* | yes [35] | [35] |
| Mulchandani-Bhoi-Conlin syndrome (MBCS) | 617352 | 20 | upd(20)mat | NR | NR | NR | NR | | [36] |

*Unexpected findings; see Ref. [46].

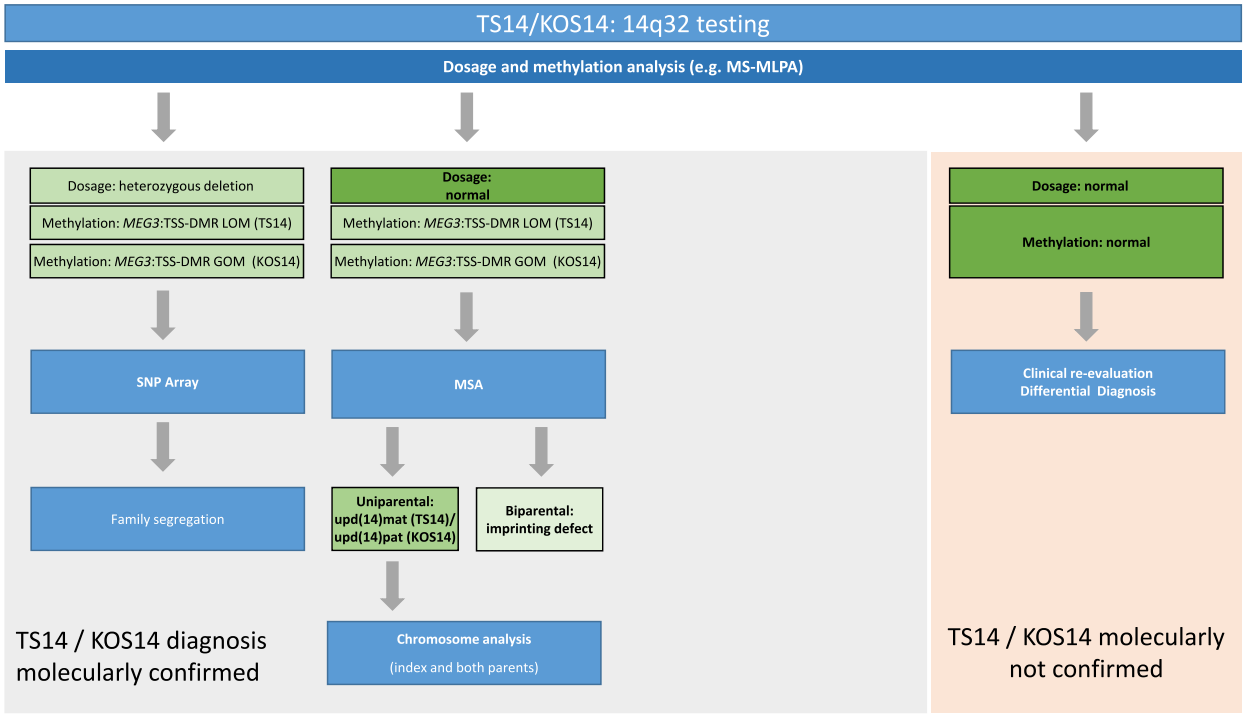
**In the case of MLID: maternal effect variants in SMC proteins have been reported, predisposing for recurrent miscarriages and MLID in the children.

***Affected parental allele associated with clinical features is listed.

****Frequencies of (epi)mutations in SRS and BWS refer to their frequencies in clinically defined cohorts, whereas in the other disorders the frequencies refer to molecularly confirmed cases.

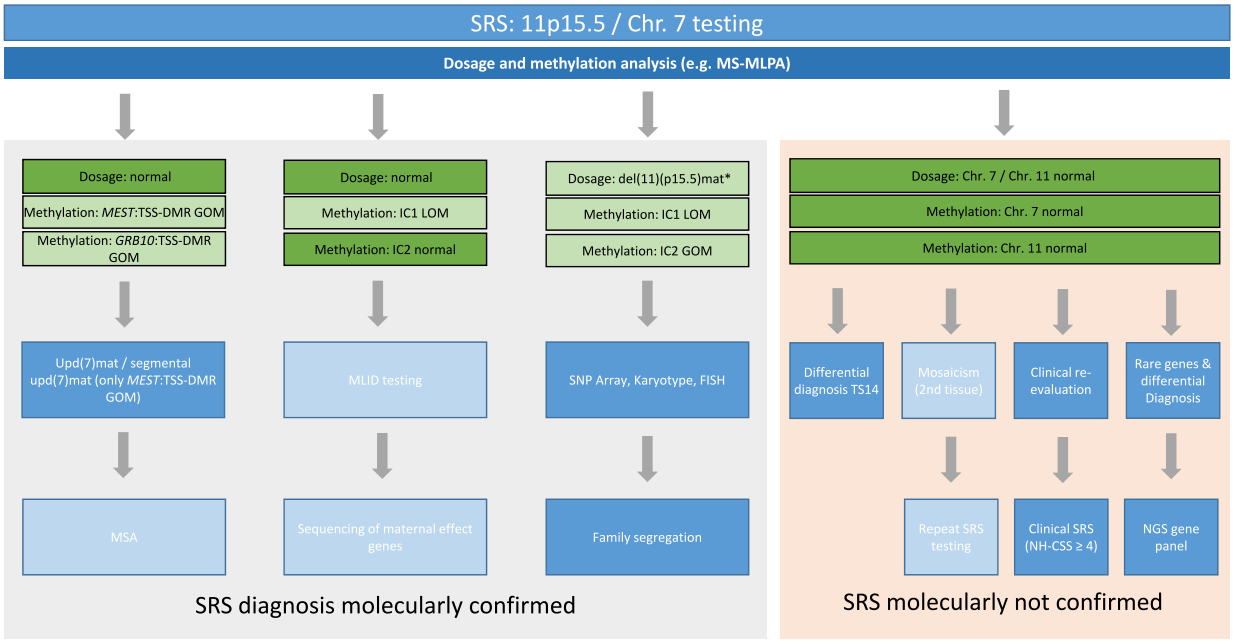



(a)

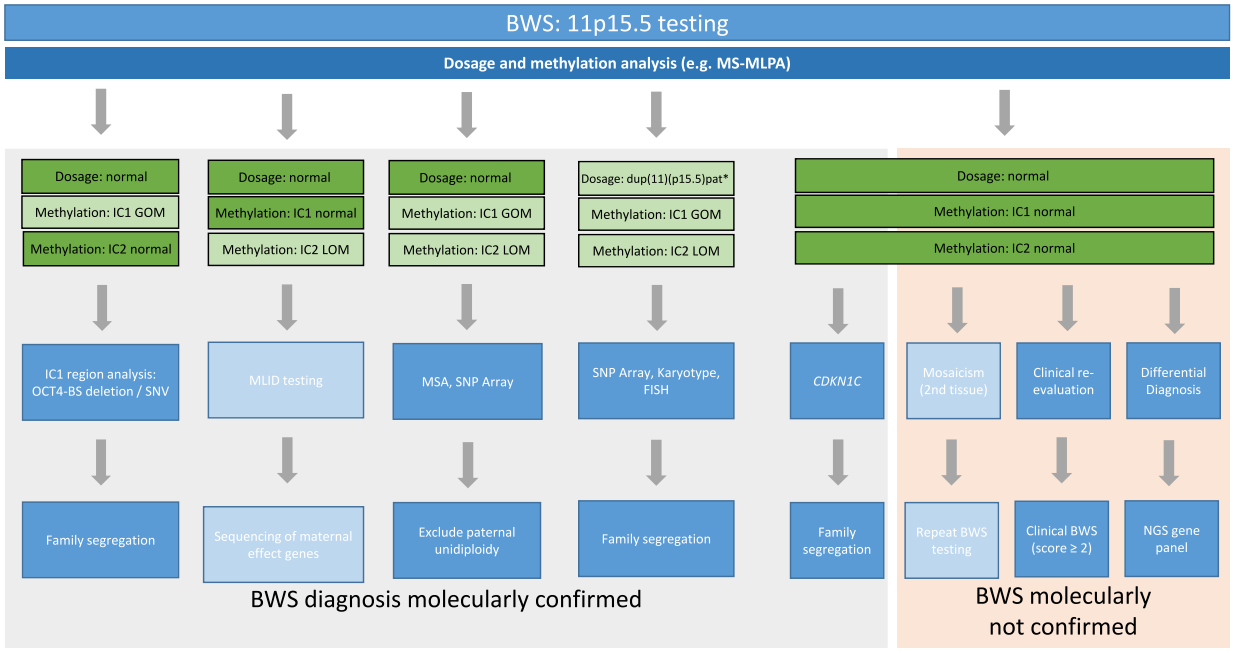



(b)

**Figure 1:** Molecular diagnostic algorithm for the four most frequently tested ImpDis, i. e. (a) PWS and AS, (b) TS14 and KOS14, (c) SRS and (d) BWS. The suggested stepwise analyses reflect the frequencies of the molecular subtypes. It should be noted that for all disorders the first step are MS tests, preferably MS-MLPA. Furthermore, the workup of rare MS-MLPA results is not shown. (*In case of deletions, the subsequent steps are similar. For abbreviations, see the list of abbreviations.)




(c)




(d)

**Figure 1:** (continued).

pathogenic CNVs, UPDs and imprinting defects. As all these disturbances affect the methylation pattern at the disease-specific locus, it is recommended to start with methylation-sensitive (MS) tests. In fact, MS multiplex ligation-dependent probe amplification (MS-MLPA) should be applied as first step analysis, as it allows the simultaneous detection of all three subtypes, and at least in SRS and BWS imprinting defects can be discriminated from UPDs (see below). If the first MS test is negative, further molecular analyses should be conducted, depending on the molecular spectrum of the disease (Table 1). However, these stepwise analyses might not be suitable for specific situations: In cases referred for UPD analyses based on chromosomal findings or a precedent (family) history with an already known molecular disturbance other suitable tests can be applied.

In several rare ImpDis the molecular spectrum comprises only one type of disturbance, and therefore testing requires only specific assays, like sequencing for pathogenic SNVs in specific disorders (e. g. Birk–Barel intellectual disability syndrome [BBIDS: *KCNK9*], Schaaf–Yang syndrome [SHFYNG: *MAGEL2*] and central precocious puberty (CPPB: *MKRN3*, *DLK1*]).

The methods available for testing of ImpDis can be classified as those targeting altered methylation and those targeting both altered methylation and genomic alterations (CNVs, UPDs). However, MS assays commonly have the capacity to detect the methylation changes caused by CNVs, imprinting defects and UPDs affecting imprinted regions, though depending on the underlying technology and the chromosome analysed not all tests can discriminate between the different molecular subtypes.

In routine diagnostics, MS-MLPA has meanwhile been accepted as the most suitable method and has replaced other test systems. MS-MLPA has been introduced in 2005 [6] and kits are available for all ImpDis in which the molecular spectrum comprises imprinting defects, (gene) dosage aberrations and UPDs. Accordingly, the vast majority of diagnostic labs have established MS-MLPA as the first trial test for ImpDis (see quality assessment scheme reports of the European Molecular Quality Network [EMQN] for PWS/AS and BWS/SRS). The advantage of MS-MLPA over other molecular (MS) tests is that it enables the simultaneous detection of aberrant DNA dosage and methylation, in a single reaction. Due to a parallel copy number and methylation analysis in one MS-MLPA run, CNVs can be clearly identified and discriminated from imprinting defects and UPDs. However, with the exception of the MS-MLPA kit for BWS/SRS (ME030, MRC Holland, Amsterdam, The Netherlands), the discrimination between imprinting defects and UPDs is often not possible because the imprinted loci within the same assays and/or within the same chromosomal region (like the chromosome 15 loci) exhibit the same parental imprint. In these cases, additional tests are required, and for confirmation of UPD microsatellite analysis (MSA) is the gold standard (see below).

By MS-MLPA, up to 40 genomic sequences can be targeted in the same run. Thereby, different CpGs of the same DMR can be analysed in parallel, and false-positive hybridisation signals due to genomic variants in a probe sequence will be uncovered. Furthermore, a DNA sample only from the index patient is required at that time. The reliability of the MS-MLPA data is substantially influenced by the applied bioinformatics analysis pipelines. In fact, several tools are available (e. g. Ref. [2]), and they have significantly improved with respect to their sensitivity. However, a lab should be aware of the need to validate these tools in the case of major changes in the algorithms, in particular if in-house tools are used for diagnostics.

Though MS-MLPA is widely used and easily acquirable, a diagnostic lab has to be aware of the limitations of the technique, in particular because of the broad molecular spectrum of genomic and epigenetic changes. In particular, the mosaic distribution of imprinting defects and upd(11p)pat in BWS can hinder their detection.

While MS-MLPA has the advantage to use native DNA (see MLPA), many other MS methods like MS-PCR, MS high-resolution melting (MS-HRM), MS Sanger sequencing or MS pyrosequencing require a chemical DNA modification, which conserves the methylation information at the sequence level. Treatment with bisulphite converts unmethylated cytosines into uracil. In the subsequent analyses, uracil pairs with adenine, which corresponds to an exchange of unmethylated cytosines to thymine at the sequence level. The possibility to analyse methylation marks results from the protection of 5-methylcytosine against the bisulphite conversion. Allele-specific methylation therefore results in a sequence difference at the corresponding position of the (un)methylated CpG.

A diagnostic test based on bisulphite-modified DNA which has been widely used is methylation-specific PCR (MS-PCR). To analyse the CpG of interest three different primers are required for amplification [7]; a common primer in combination with specific primers for the methylated and the unmethylated allele are used, respectively. The allele-specific primers span several differentially methylated CpGs, and the PCR products can be discriminated by gel electrophoresis. A major limitation of MS-PCR and the other bisulphite-based tests for diagnostics is that they do not allow the discrimination between the different molecular types of ImpDis. Furthermore, as single locus PCR assays, they are prone to allelic dropout by SNVs within the primer sequences. In fact, allelic dropout might also affect the MS-MLPA hybridisation, but MLPA assays commonly include several probes for the same region and thereby enable the detection of allelic dropout.

## Microsatellite analysis

MSA is the gold standard for testing for UPD. MSA utilises highly variable microsatellites (also referred to as short tandem repeats) to establish the parental origin of the chromosomes and to differentiate between isodisomy and heterodisomy which points to the underlying mechanism of UPD formation (e. g. monosomy or trisomy rescue, respectively). Usually, several markers that are located

within and close to the imprinted region of interest (e. g. 15q11q13, 14q32 or 11p15.5) and some located more or less equally distributed along the chromosome of interest are investigated in the patient and both parents. In fact, reporting of two informative markers has been suggested as sufficient [8]. UPDs can occur for a whole chromosome or only for a certain segment (segmental UPD).

Some labs use SNP array analyses for UPD testing. These labs need to be aware that if only the patient is tested one cannot exclude a heterodisomy and thus might miss a substantial number of UPDs. It is recommended to analyse the patient as well as both parents by SNP array to be able to make a clear diagnosis.

## Reporting of molecular test results in imprinting disorders

The format of the reports on ImpDis testing should correspond to international and national guidelines (e. g. Association for Clinical Genetic Science, British Society for Genetic Medicine). It is recommended that testing laboratories are accredited to international standards, for example, DIN EN ISO 15189 or equivalent. Laboratories reporting imprinting test results should participate annually in external quality assessment if available, and the scheme should cover both wetlab and reporting. In addition to general format guidelines, the reports should include the following items:

- A consistent naming of the analysed DMRs should be used in the same report, and recently a common nomenclature has been published [9]. The nomenclature of genomic pathogenic variants has to be in accordance with the suggestions of the Human Genome Variation Society (HGVS) (http://www.hgvs.org/mutnomen/). Of note, the HGVS and ISCN nomenclatures are not suitable for reporting MS-MLPA results.
- Applied methods and a statement on the limitations and sensitivities including detection of low-grade mosaicism should be included. Appropriate references should be given, and if commercial kits are used, the kit version has to be mentioned (for example, ME030-C3, http://www.mrc-holland.com). For MS-PCR the respective paper needs to be cited,
- the precise result description (if present, description of aberrant methylation [loss of methylation (LOM), gain of methylation (GOM)], CNVs; precise delineation of the deleted or duplicated region including genomic positions and genome build or the names of the MLPA probes),
- an interpretation of diagnostic testing results,
- a clear statement whether the clinical diagnosis is confirmed or not,
- depending on the in-house policy, a statement on the significance of the molecular results for the clinical management, based on the specific guidelines if available (Table 1),
- in case the results of only single steps of the diagnostic algorithm are reported, the consecutive steps must be suggested,
- genetic counselling has to be recommended, precise recurrence risks have to be given if possible (see also Elbracht et al. in this issue).

## Molecular findings in specific imprinting disorders and relevance for diagnostic testing

Whereas in some ImpDis only single types of molecular disturbances can be observed, the molecular spectrum of the more prominent entities comprises almost all four classes of changes (Table 1). In fact, the first group of disorders consists of the recently defined ImpDis characterised by pathogenic SNVs in single genes. However, as recent reports on TS14 and KOS14 show, it can be expected that the spectrum of molecular disturbances might expand in at least some of them.

In the following, we will focus on major molecular aspects of the most frequently diagnosed ImpDis, but the reader should be aware that the knowledge on the molecular basis of ImpDis is dynamic and requires a permanent follow-up.

### The chromosome 15q11q13-associated imprinting disorders: Angelman syndrome and Prader–Willi syndrome, Schaaf–Yang syndrome, central precocious puberty 2 (MKRN3)

The 15q11q13 region is imprinted as it harbours a differentially methylated imprinting centre (*SNURF*:TSS-DMR) which is paternally unmethylated and maternally methylated [10]. This results in differential expression of the following genes in the region: *NDN*, *MAGEL2*, *MKRN3*, *NPAP1*, *SNURF-SNRPN* and several snoRNAs are paternally

expressed, whereas *UBE3A* is maternally expressed in the brain only (Figure 2).

The molecular causes of AS and PWS are a 5–7 Mb deletion in 15q11q13, a UPD or an imprinting defect which is mostly sporadic without a DNA sequence change [11, 12]. However, in 10–15 % of all imprinting defect cases the defect is due to an imprinting centre (IC) deletion in 15q11.2 [13]. The 5–7 Mb deletions are defined as class I and class II deletions, which range from BP1 to BP3 and BP2 to BP3, respectively (for review, see Ref. [3]).

The first step in AS and PWS diagnostics is the MS-MLPA analysis, which allows simultaneous analysis of methylation and dosage at 15q11q13 (Figure 1; see also Ref. [3]).

In AS, hypomethylation (complete LOM or methylation mosaic – see below) is detected in about 80 % of cases. This confirms the diagnosis of AS. However, in about 20 % of cases methylation is normal and sequence analysis of the *UBE3A* gene is indicated [14]. In PWS, hypermethylation (complete methylation) is detected in about 99 % of cases, which confirms the diagnosis of PWS.

In case of a deletion no methylation (AS) or complete methylation (PWS) is detectable as the methylated maternal or unmethylated paternal allele is deleted. Dosage analysis reveals whether the molecular cause is a large heterozygous deletion in 15q11q13 (class I/class II deletion) – corresponding probes will show a 50 % reduction (Table 1; see also Refs. [3, 11, 12]). In rare cases the deletion is larger and extends beyond BP3, which is indicated by the telomeric gene *APBA2*. In this event the size of the deletion should be further characterised by an appropriate method, e. g. SNP-array. If the molecular cause is a class I/class II deletion, molecular cytogenetic analysis (FISH) for the *SNRPN* locus in the index and the mother (AS)/father (PWS) is recommended to exclude the possibility of a cryptic balanced translocation which would increase the recurrence risk (for review, see Refs. [15, 3]). In case of a upd(15) both chromosomes are inherited from one parent and show either no methylation (upd(15)pat, AS) or complete methylation (upd(15)mat, PWS). Dosage analysis is normal as two alleles are present. The same result, no methylation (AS)/complete methylation (PWS) and normal dosage, is obtained in the case of an imprinting defect without an IC deletion. However, this time chromosomes 15 are inherited biparentally, but the maternal chromosome 15 carries a paternal imprint and is therefore unmethylated (AS) or rather the paternal chromosome 15 carries a maternal imprint and is therefore methylated (PWS). To distinguish between a upd(15) and an imprinting defect without an IC deletion, MSA has to be performed for the patient and both parents. In the case of a upd(15) only paternal alleles (AS)/maternal alleles (PWS) will be detected in the patient [3]. Detection of heterodisomy or isodisomy or rather a mixture of both due to cross-over events is possible. If the molecular cause is a upd(15), cytogenetic analysis in the patient and both parents is recommended to exclude the possibility of a Robertsonian translocation which would increase the recurrence risk.

Some AS patients with an imprinting defect (without an IC deletion) however show partial hypomethylation. In these cases, the imprinting defect is present in a mosaic state (cells with normal methylation and cells with an imprinting defect are present) and therefore has to have occurred after fertilisation [16].

An imprinting defect can be sporadic without any detectable deletion or DNA sequence changes at the IC region or it can be due to an IC deletion [17]. In this case dosage analysis by MLPA in the mother (AS)/father (PWS) is recommended to estimate the recurrence risk. If the mother (AS)/father (PWS) is a heterozygous carrier of the IC deletion the recurrence risk is 50 %, while for an imprinting defect without an IC deletion it is <1 % [13]. However, germ line mosaicism for the IC deletion cannot be excluded. Therefore, prenatal diagnostics should be offered for further pregnancies [10]. Where required, other family members should also be tested, since IC deletions can be inherited silently over several generations.

In cases where the MS-MLPA reveals normal results for methylation and dosage but there is still a clinical suspicion of AS, sequence analysis of the *UBE3A* gene should be performed as in 5–10 % a mutation can be detected [14]. In this event it should be clarified whether the mutation is *de novo* or inherited from the mother. If the mother is a heterozygous carrier of the *UBE3A* mutation, there is a 50 % recurrence risk and additional maternal family members should be tested as the mutation can be inherited silently over several generations. However, as the possibility of a germ line mosaic cannot be excluded, prenatal testing should be offered for future pregnancies.

In cases where PWS could not be confirmed as the MS-MLPA reveals normal results for methylation and dosage other differential diagnoses, e. g. TS14 and SHFYNG, should be considered [18, 19].

Patients with SHFYNG show a phenotype that strongly overlaps with PWS, the most prominent additional feature being arthrogryposis. In 2013 Schaaf and colleagues identified truncating mutations on the active paternal allele within the *MAGEL2* gene as causative [19]. Since then several additional patients have been described, all carrying

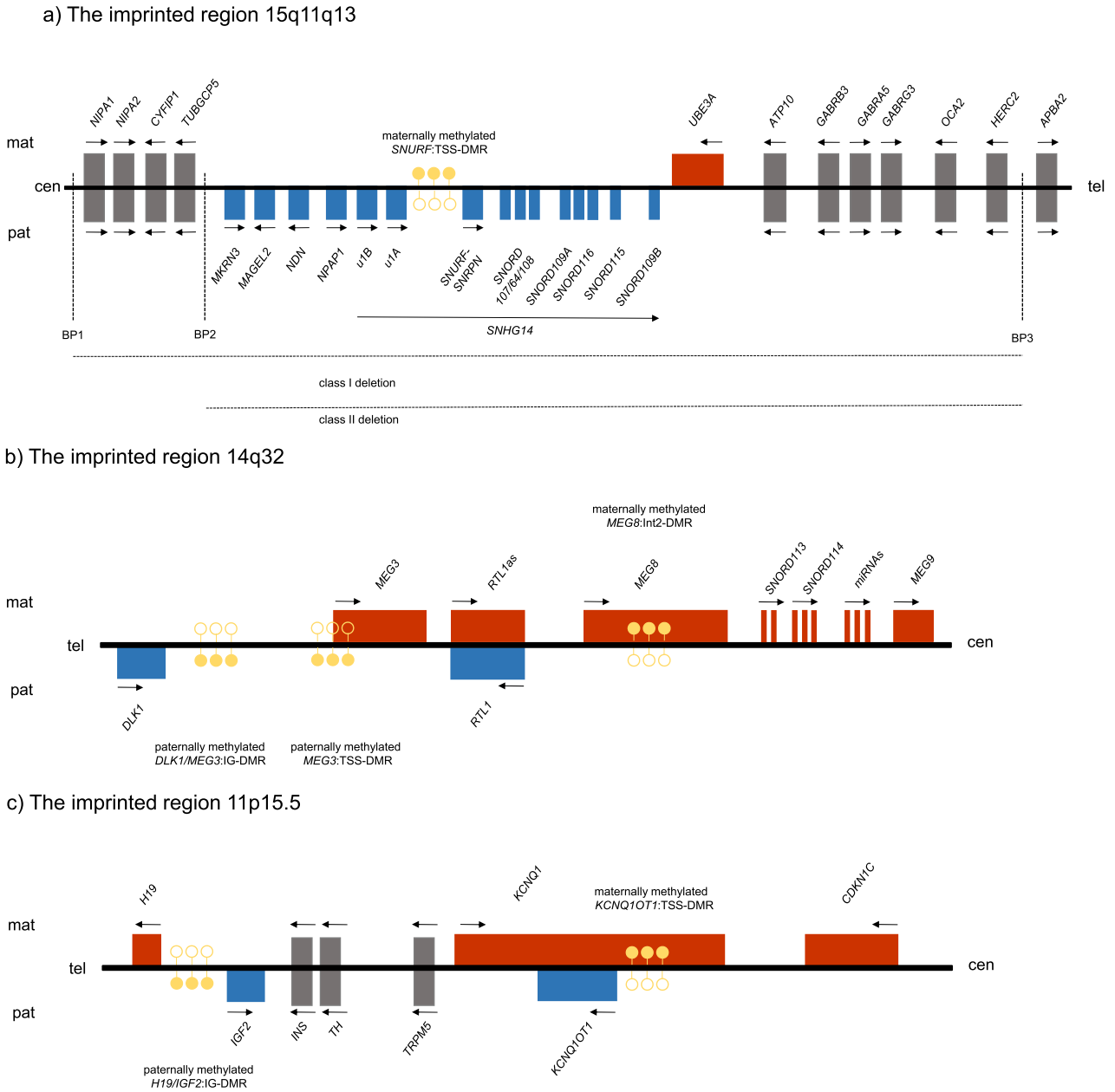



**Figure 2:** Schematic illustration of the imprinted chromosomal regions (a) 15q11q13, (b) 14q32 and (c) 11p15.5. Alterations of these imprinting clusters are associated with the most frequently diagnosed ImpDis. They all include characteristic elements of imprinting domains, i. e. differentially methylated regions (DMRs) exhibiting methylation (filled lollypops) on either the maternal or the paternal allele, and non-coding RNAs (e. g. *KCNQ1OT1* on 11p15.5, *SNORD116* on 15q11q13, *MEG3* on 14q32). The DMRs regulate genes with a parent-of-origin monoallelic expression (shown by an arrow; red, maternally expressed; blue, paternally expressed; grey, biallelic expression; BP, breakpoint cluster).

truncating mutations (e. g. Ref. [20]). Of note, deletions affecting the whole *MAGEL2* gene on the paternal allele do not cause SHFYNG [21].

For molecular genetic diagnostics of SHFYNG the *MAGEL2* gene is sequenced. If a truncating variant is identified, it should be verified that it resides on the active, paternal allele. This is usually done by family analysis. If the father is carrier of the truncating variant, investigation of additional paternal family members should ensue as the mutation can be transmitted silently over generations. If the father does not carry the mutation or is unavailable, methylation-specific restriction followed by a matched PCR should be performed to identify the variant-carrying allele.

Mutations on the active paternal allele of the imprinted gene *MKRN3* in 15q11.2 result in CPPB2 [22]. In families with *MKRN3*-associated precocious puberty, both sexes are affected, and pathogenic variants are inherited from the father. Therefore, family analysis should be performed to determine the parental status of a detected *MKRN3* mutation.

## The chromosome 14q32-associated imprinting disorders: Temple syndrome and Kagami–Ogata syndrome

The chromosomal region 14q32 harbours a cluster of imprinted genes which expression is regulated by two imprinting control regions, i. e. the primary, paternally methylated *MEG3-DLK1*:IG-DMR and the secondary, also paternally methylated *MEG3*:TSS-DMR (Figure 2c). Additionally, the region harbours a third DMR, *MEG8*-Int2:DMR, which is also secondarily derived but, in contrast to the other two, is methylated on the maternal allele. Genetic and epigenetic disturbances within 14q32 lead to two distinct phenotypes: TS14, which is characterised by hypotonia in early childhood, short stature, obesity and early puberty and shows a clinical overlap with PWS and SRS, and KOS14, with its most prominent phenotypic feature being a bell-shaped thorax and coathanger-like ribs, leading to respiratory insufficiency and feeding difficulties (for clinical details, see Elbracht et al. in this issue).

The molecular genetic causes of TS14 and KOS14 include upd(14), imprinting defects and deletions of various sizes [16, 23, 24, 25]. UPDs, imprinting defects and some deletions can be detected by methylation analysis of *MEG3*:TSS-DMR. The methylation at the *MEG3-DLK1*:IG-DMR is not suitable for diagnostic testing [26]. Other deletions that do not affect the methylation have been described and can be detected by MS-MLPA or other dosage analyses (see below).

The best-suited method for diagnostic testing at the moment is MS-MLPA (Figure 1). The commercially available MS-MLPA kit (ME032, MRC Holland) currently contains three MS probes for the *MEG3*:TSS-DMR as well as 12 probes for dosage analysis in *DLK1*, *MEG3*, *RTL1* and *MIR*380.

Hypomethylation (LOM) of the *MEG3*:TSS-DMR on the paternal allele leads to loss of expression of the paternally expressed genes and TS14. On the other hand, complete methylation (GOM) of the *MEG3*:TSS-DMR causes a loss of expression of the maternally expressed genes and thereby KOS14 [16, 22].

When the dosage analysis is normal, LOM/GOM can be either due to a upd(14)mat in the case of TS14 or a upd(14)pat in the case of KOS14, or an imprinting defect which can also be present in a mosaic state. To discriminate between these two molecular causes, MSA in the patient and both parents needs to be performed. If a upd(14) is confirmed, chromosome analysis in the patient and both parents should ensue to rule out a Robertsonian translocation as this would increase the recurrence risk.

In TS14 (mosaic) imprinting defects have been reported in a growing number of cases (e. g. Ref. [27]). But also maternal and paternal upd(14) and deletions on the maternal or paternal allele have been reported in a mosaic state. Furthermore, there have been reports of upd(14) associated with supernumerary marker chromosomes or mosaic trisomy 14. Therefore, in case a chromosome analysis shows an aberration involving chromosome 14, upd(14) testing needs to be considered.

There are several reports on deletions of different sizes and affecting different parts of the region that lead to either TS14 or KOS14. The deletions can vary in size, ranging from only a few kb to 1.1 Mb (see below). They can comprise one or both of the imprinting control regions or they can encompass none of them and thus do not affect the methylation. In some cases, these deletions can be transmitted silently over generations; in other cases, the deletion will lead to TS14 or KOS14, depending on the transmitting parent, in every generation (e. g. Refs. [26, 28]). Therefore, it is of utmost importance to characterise the detected deletions with suitable methods and to investigate the respective family members to determine the recurrence risk. If all probes within the MS-MLPA kit show a heterozygous deletion this could be due to the recurrent 1.1 Mb deletion that has been described in TS14 patients with an additional phenotype of developmental delay and intellectual disability which is due to haploinsufficiency of the gene *YY1* [29].

Due to the clinical overlap, TS14 testing should be considered in patients with features of SRS or PWS who have no molecular confirmation. Recently, pathogenic variants on the active, paternal allele of the *DLK1* gene have been identified in cases of precocious puberty [30].

## The chromosome 11p15.5-associated imprinting disorders: Silver–Russell syndrome and Beckwith–Wiedemann syndrome

Two of the major imprinted regions in humans are localised on the short arm of chromosome 11 (11p15.5). The

chromosomal region 11p15.5 harbours two separate ICs (Figure 2b). The telomeric IC1 includes the *H19/IGF2*:IG-DMR, which is methylated on the paternal allele, while the centromeric IC2 consists of the maternally methylated *KCNQ1OT1*:TSS-DMR. Several of the 11p15.5 genes are involved in human growth and development as well as in tumourigenesis. As a result, SRS and BWS as the two ImpDis associated with 11p15.5 alterations are clinically characterised by either growth retardation (SRS) or overgrowth (BWS), dysmorphisms and, in the case of BWS, an increased risk for tumours (for further clinical details, see Refs. [4, 5] and Elbracht et al. in this issue).

The types of mutations and imprinting defects in SRS and BWS affect the two ICs in an opposite manner (Table 1) (for review, see Refs. [4, 5]) and with different frequencies, but molecular testing for both disorders should start with MS-MLPA (Figure 1c, d). The majority of patients with the typical SRS phenotype carry an LOM of the *H19/IGF2*:IG-DMR (for review, see Ref. [4]). Between 4 % and 10 % of SRS patients carry maternal UPD of chromosome 7 (upd(7)mat); therefore, analysis of upd(7)mat is mandatory in patients referred for SRS testing. A growing number of patients referred for SRS testing exhibit molecular variants in 14q32, thus having molecularly confirmed TS14, which is a differential diagnosis for SRS. SNVs in the 11p15.5-encoded genes *CDKN1C* (gain-of-function variants at certain codons) and *IGF2* are rare and have mainly been reported in familial cases. Additionally, in a considerable number of patients (submicroscopic) alterations of chromosomes other than 7 and 11 may be detected [31]; therefore, molecular karyotyping is indicated. In single cases, maternal UPD of chromosomes 16 and 20 (upd(16)mat, upd(20)mat) has been reported.

With the exception of patients with *H19/IGF2*:IG-DMR LOM, the clinical findings in carriers of the other molecular changes do not always fit the clinical Netchine–Harbison scoring for SRS [4]. In particular in neonates and adults the decision on molecular testing cannot always be based on a convincing phenotype, thus testing might also be applied to patients with less obvious growth parameters and dysmorphic signs.

In BWS, the most frequent change is the LOM of *KCNQ1OT1*:TSS-DMR, accounting for up to 50 % of patients (Table 1) (for review, see Ref. [5]). The second most frequent alteration in BWS is upd(11)pat, detectable in nearly 20 %. The *H19/IGF2*:IG-DMR is affected by GOM 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 IC1 cause the aberrant methylation [32]. Deletions, duplications and even balanced translocations in 11p15.5 [33, 34, 35] also contribute to the mutational spectrum, with the size and parental origin of the affected region influencing the phenotype. Loss-of-function variants in *CDKN1C* are not only frequent in familial cases but are also of importance in sporadic BWS with a frequency of 5 %. Familial cases mainly present with *CDKN1C* mutations (50 %), chromosomal duplications/deletions or mutations in other genes/regulative elements (e. g. *OCT4/SOX2*, CTCF bindings sites).

For molecular diagnostic testing it is important to keep in mind that the predominant alterations in both disorders, *H19/IGF2*:IG-DMR hypomethylation in SRS, as well as the upd(11)pat and 11p15 imprinting defects in BWS, often occur in a mosaic state.

## The chromosome 20q13-associated and further imprinting disorders

Though it is rare, pseudohypoparathyroidism 1B (PHP1B) is one of the classical ImpDis and is a molecular subentity of PHPs (for review, see Ref. [36]). These disorders are characterised by parathyroid hormone (PTH) resistance in the kidney. Most cases of PHP belong to type 1, i. e. are caused by genetic or epigenetic alterations at the imprinted *GNAS* locus. PHP1A is caused by inactivating mutations in the maternal allele of the *GNAS* gene. Paternal *GNAS* mutations are associated with Albright hereditary osteodystrophy (AHO), no hormonal resistance and no obesity (pseudo-PHP) as well as with progressive osseous heteroplasia. In contrast, the phenotype of most PHP1B patients is limited to renal PTH resistance. Few patients with PHP1B display some features of AHO. Patients with PHP1B share an LOM at the *GNAS A/B*:TSS-DMR of *GNAS*. Some patients carry additional epigenomic changes along the *GNAS* locus. About 20 % of PHP1B cases are inherited and due to deletions of *GNAS* imprinting control regions. The remaining 80 % are sporadic. A small subset are due to paternal UPD of chromosome 20q.

Maternal UPD of the same region (upd(20)mat) has recently been established as Mulchandani–Bhoj–Conlin syndrome (MBCS) [37]. It describes a growth retardation phenotype but without specific further features. The majority of cases have so far been discovered in patients referred for SRS testing; therefore, upd(20)mat testing should be considered in this clinical cohort.

Birk–Barel syndrome (BBIDS) is characterised by moderate to severe intellectual disability, hypotonia and facial dysmorphism. The molecular causes are missense mutations in the maternally expressed gene *KCNK9* located in the chromosomal region 8q24.3. So far only two

highly conserved amino acid positions (mainly Pro-236 and Pro-237) have been reported to be affected [38]. The only other imprinted gene in the region is the paternally expressed *PEG13*, which has a maternally methylated DMR (*PEG13*:TSS-DMR) that exhibits brain-specific enhancer-blocking/insulator activity, thus regulating the imprinted expression of *KCNK9* [39]. However, no methylation disturbance causing Birk–Barel syndrome has been reported until now. Therefore, diagnostic testing consists of sequencing of the *KCNK9* gene.

## Multilocus imprinting disturbances

Though the association between molecular disturbances at specific imprinted genes and distinct congenital disorders is well known (Table 1), the number of reports on patients with general methylation disturbances (MLID) is increasing (for review, see Ref. [40]).

MLID can be observed in nearly 50 % of transient neonatal diabetes mellitus (TNDM) patients with LOM of the *PLAGL1*:alt-TSS-DMR, in nearly 25 % of BWS patients with LOM of the *KCNQ1OT1*:TSS-DMRs and in 7–10 % of SRS patients with hypomethylation of the *H19*/*IGF2*:IG-DMR. In SRS, PHP1B and TS14, MLID has been reported as well, but not for AS or PWS. Carriers of MLID often exhibit a specific ImpDis phenotype, e. g. BWS, but at least for TNDM clinical differences to other molecular subgroups are obvious [41]. Thus, a comprehensive documentation of MLID phenotypes is required in the future to overview its clinical spectrum. A common cause of MLID has not yet been reported, but mutations in trans-acting genes/factors as well as maternal effect genes have been identified in some cases (for review, see Ref. [40]). Thus, whole exome/genome sequencing strategies or candidate gene analyses might be considered in these patients and their parents on a research basis and in cooperation with reference centres. The identification of MLID might not only be relevant for its carrier in the future, but it has already been shown that it might be associated with reproductive failure in the families (for further details, see Elbracht et al. in this issue).

## Mosaicism as diagnostic challenge and explanation for a diagnostic gap

Whereas mosaicism does generally not occur in the case of CNVs or SNVs, it can be observed in carriers of imprinting defects and upd(11)pat in BWS (Table 1). In particular, mosaicism has to be considered in diagnostic testing of BWS, SRS, AS, TS14 and KOS14. Low-level mosaicism within a tissue may not be detected because of the detection limit of the methods used, thereby leading to false-negative results. As it has been shown recently for SRS [42], the fraction of aberrant cells may vary significantly among different tissues. After clinical reevaluation testing other tissues should therefore be discussed to exclude tissue-specific mosaicism.

## Prenatal testing of imprinting disorders

The growing knowledge on the molecular basis of ImpDis and the increasing availability of MS-MLPA assays results in an increasing demand for prenatal testing of ImpDis. However, prenatal molecular testing for these entities is complex and raises numerous questions ranging from methodological questions (limitations and information value) to ethical topics. These issues have to be addressed before a prenatal test is offered and have to be discussed between the families, the genetic counsellors, the obstetrics and the laboratory (for further details, see Ref. [43]).

For AS and PWS prenatal testing can be offered as the imprint at the *SNURF*:TSS-DMR is already set and stable in chorionic villi and amniotic fluid samples. However, one should be aware that the methylation at the *MAGEL2*:TSS-DMR and the *NDN*:TSS-DMR is not fully established in chorionic villi and amniotic fluid samples. Therefore, only the methylation status of *SNURF-SNRPN* should be considered for prenatal testing [10].

For chromosome 14 methylation data on the *MEG3*:TSS-DMR in prenatal tissues are sparse [44]. Therefore, prenatal testing is done based on MSA in case one of the parents is a carrier of a Robertsonian translocation involving chromosome 14. In case of deletions or chromosomal aberrations where only TS14 could be the consequence, the mild phenotype of TS14 has to be considered and the risks and consequences of such a test should be critically weighed.

Prenatal testing for SRS and BWS should consider these limitations as well, and in both disorders the (negative) prenatal testing result is only of limited reliability [43].

## Outlook

Though targeted testing in case of specific phenotypes is often recommended to avoid ambiguous and incidental

findings, the broad molecular spectrum of genomic and epigenetic alterations shows that in patients with ImpDis multilocus testing might be indicated. Recent rapid advances in the identification of molecular disturbances in ImpDis and the identification of new entities have illustrated the complexity of imprinting regulation, and therefore its molecular and clinical diagnosis. In fact, the precise identification of (new) mutational and epimutational pathways offers the potential for new therapeutic regimes as the basis for a more directed and personalised medicine in imprinting disorders (see Horsthemke and Zechner in this issue).

The application of more sensitive and high-throughput methods (i. e. next- and third-generation sequencing) will further enlighten the molecular spectrum of alterations in ImpDis, help to reduce the problem of mosaicism (e. g. Ref. [45]) and contribute to the understanding of imprinting regulation. As the recent identification of new ImpDis shows (i. e. CPPBs, MBCS), these strategies help to discover new entities, and it will be interesting to see whether (epi)genetic constitutions like upd(6)mat or upd(16)mat will turn out to present ImpDis.

## Website links

Association for Clinical Genetic Science, British Society for Genetic Medicine:
http://www.acgs.uk.com/media/949852/acgs_general_genetic_laboratory_reporting_recommendations_2015.pdf
GeneReviews:
https://www.ncbi.nlm.nih.gov/books/NBK1116/

## List of Abbreviations

| | |
|---|---|
| AHO | Albright hereditary osteodystrophy |
| AS | Angelman syndrome |
| BBIDS | Birk–Barel mental retardation syndrome |
| BWS | Beckwith–Wiedemann syndrome |
| CPPB2 | Central precocious puberty 2 |
| DMR | Differentially methylated region |
| FISH | Fluorescence in-situ hybridisation |
| GOM | Gain of methylation, hypermethylation |
| IC | Imprinting centre |
| ImpDis | Imprinting disorder |
| KOS14 | Kagami–Ogata syndrome |
| LOM | Loss of methylation, hypomethylation |
| MBCS | Mulchandani–Bhoj–Conlin syndrome |
| MLPA | Multiplex ligation-dependent probe amplification |
| MS | Methylation-sensitive |
| MSA | Microsatellite analysis |
| MS | HRM Methylation-sensitive high-resolution melting |
| PTH | Parathyroid hormone |
| PWS | Prader–Willi syndrome |
| PHP1B | Pseudohypoparathyroidism type 1b |
| SHFYNG | Schaaf–Yang syndrome |
| SRS | Silver–Russell syndrome |
| TNDM | Transient neonatal diabetes mellitus |
| TS14 | Temple syndrome |
| UPD | Uniparental disomy |
| upd(15)mat | Maternal uniparental disomy of chromosome 15 (similar description for other maternal or paternal UPDs) |

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