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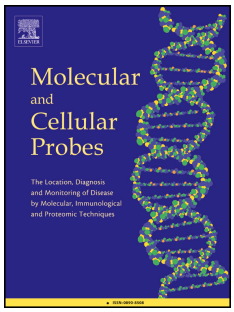

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Congenital Imprinting disorders: Application of multilocus and high throughput methods to decipher new pathomechanisms and improve their management

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Abstract

Imprinting disorders (IDs) are a group of congenital diseases affecting growth, development and metabolism. They are caused by changes in the allele-specific regulation ("epigenetic mutation") or in the genomic sequence ("genetic mutation") of imprinted genes. Currently molecular tests in ID patients are generally restricted to single loci classically associated with the disease, but this approach limits diagnostic yield, because of the molecular and clinical heterogeneity between IDs. From the technical point of view, these limitations are aggravated by the lack of standardization in testing methodology, in the DNA sequences tested, and in clinical inclusion criteria prompting testing.

However, an increasing number of new studies show that these problems can be addressed by the use of new tests targeting multiple loci and/or a total exome and genome analysis.

The rapid development of efficient and high-throughput molecular techniques and their applications in research and diagnostics in the last decade have led to an impressive increase of knowledge on IDs and their basic pathomechanisms. In combination with the improvement of data recording and documentation, the diagnostic strategies are increasingly based on standardized protocols, and thereby provide the backbone for directed counselling, more personalized management, and new therapeutic approaches.

Keywords: Imprinting Disorders – methylation-specific assay – multilocus imprinting analysis – high throughput techniques

1. Introduction

Imprinting disorders (IDs) are a group of congenital diseases affecting growth, development and metabolism characterised by similar molecular alterations (Table 1).

They are caused by changes in the allele-specific regulation ("epigenetic mutation") or in the genomic sequence ("genetic mutation") of imprinted genes and regions, respectively (Figure 1). In contrast to the majority of biallelically expressed genes, imprinted genes are expressed monoallelically in a parent-of-origin specific manner - i.e. either from the maternal or the paternal allele (for review: [1]). At the molecular level, the expression of genes within imprinted regions is influenced by specific patterns of DNA methylation, changes in chromatin structure, and post-translational histone modifications, collectively designated as epigenetic regulation (for review: [2, 3]). So far, more than 90 human genes have been confirmed to be imprinted, but there are probably more based on bioinformatics predictions (for review: http://www.geneimprint.com/site/home, [www.geneimprint.com/site/genes-by-species] last check: 19.04.2015). The normal imprinting marks are inherited from the parental gametes and are then maintained in the majority of somatic cells and tissues of an individual. Their programming is subject to an imprinting cycle during life which leads to a reprogramming at each generation (for review: [4, 5]). Methylation of the mammalian genome is comprehensively remodelled in early development. However, imprinting marks are exempted from developmental reprogramming; instead, they are erased in the germ-line and re-established according to the sex of the contributing parent for the next generation. Many genes regulated by genomic imprinting are found in clusters, i.e. imprinted loci often comprise multiple genes under coordinated control. A prominent example is the chromosomal region 11p15.5 which harbors genes encoding several growth promoting and inhibiting factors. It spans around 1 Megabase (Mb) and maintains two separate imprinting control regions (ICRs): the telomeric imprinting control region 1 (ICR1; H19 differentially methylated region - DMR) is methylated on the paternal allele, whereas the centromeric ICR2 (KvDMR1; KCNQ1OT1 DMR) is maternally methylated. In addition to its central physiological role in human growth and development it has been postulated that the 11p15.5 region is a central element of a network of imprinted genes [6, 7].

2. Imprinting Disorders (IDs): (epi)genetic aetiology and phenotypes

ID patients carry molecular disturbances which result in an unbalanced expression of imprinted genes. So far, four different types of alterations have been reported (Figure 1), (i) uniparental disomy (UPD), (ii) deletions or duplications of the imprinted region, (iii) aberrant methylation marks (called epimutations), and (iv) point mutations in (imprinted) genes (see below). It is assumed that these (epi)mutations cause unbalanced expression of imprinted genes and thereby the clinical features of IDs, but functional proof of this is lacking in the majority of IDs.

In several IDs, two additional molecular features may be present: (a) mosaic distribution of epimutations and UPD, i.e. not all cells carry the disturbance causative of disease; and (b) the occurrence of multilocus imprinting defects (MLID) in a proportion of patients with epimutations (table 1)

For the majority of the known IDs and their molecular defects, the pathophysiological mechanisms resulting in the specific phenotypes are unknown. So far only three genes have been shown to be directly associated with clinical phenotypes (Table 1): *CDKN1C* in Beckwith-Wiedemann syndrome and Silver-Russell-Syndrome (BWS, SRS), *UBE3A* in Angelman syndrome (AS) and *MKRN3* in central precocious puberty [8]. These genes are themselves imprinted; as a result, the inheritance of mutations in *UBE3A* and *CDKN1C* is autosomal dominant but its penetrance depends on the sex of the contributing parent.

The impact of genomic mutations in imprinted genes on the clinical outcome has furthermore been shown for mental retardation: maternally inherited *KCNK9* mutations have been identified to cause the Birk-Barel mental retardation syndrome [9].

An indirect cause of IDs is the mutation of genes encoding factors involved in the establishment and maintenance of imprinting. One such factor is the *ZFP57* gene mutations in which cause MLID and is inherited in an autosomal recessive manner. Interestingly, ZFP57 mutations have never been except in association with clinical presentation of transient neonatal diabetes mellitus (TNDM), An additional group of factors associated with aberrant methylation are the *NLRP* genes, but mutations in these genes act as maternal effect mutations, that means the maternal genotype causes aberrant methylation in the offspring [10, 11].

So far, ten IDs have been reported (Table 1) and the clinical and molecular definition of further disorders is a matter of active discussion. The majority of IDs share major clinical characteristics, i.e.:

- pre- and/or postnatal growth retardation or pre- and postnatal overgrowth;
- hypo- or hyperglycemia;
- abnormal feeding behavior in early childhood and later
- behavioral difficulties (in childhood)
- precocious puberty.

Nearly all patients with an ID are diagnosed in (early) childhood. However, clinical diagnosis is often hampered by the breadth of the phenotypic features which are sometimes subtle, overlapping and transient; this latter point in particular can obscure diagnosis in puberty and adulthood. As a result some IDs and a proportion of patients are probably either mis- or undiagnosed.

3. Currently applied single-locus tests and their limitations

Up to now, molecular tests in ID patients are generally restricted to single disease-specific loci. Thus, the detection rates for epimutations or mutations at these loci are more or less well established. However, technical, biological and clinical factors influence the diagnostic yield in IDs, and thereby limit the diagnostic detection rates.

3.1 Limitation 1: lack of standardization of the applied tests

A broad range of molecular techniques with different sensitivities is applied in diagnostic testing of IDs (Table 2), and they often target different differentially methylated CpG dinucleotides and even different differentially methylated regions (Figure 2). Due to this lack of standardization, it is difficult to compare the molecular results between different studies or laboratories offering molecular diagnosis for the same disorder. However, as data for the GNAS locus reveals, the heterogeneity of molecular tests affects the detection of mosaic (epi)mutations rather than the correctness of the molecular diagnosis [12].

3.2 Limitation 2: Heterogeneity of the aetiology in IDs

The heterogeneity of molecular aetiology in IDs has become apparent with the increased use of methods capable of parallel detection of mutations and epimutations at different loci

(multilocus methylation tests, array typing, Next Generation Sequencing/NGS; Table 3). Recently, we reported on our results from a cohort of 711 patients referred with the clinical diagnosis of one of the two chromosome 11p15-associated IDs, SRS (n=571) or BWS (n=140). Molecular testing was not restricted to the 11p15 imprinted loci, but also encompassed differentially methylated regions (DMRs) on chromosomes 6, 7, 14 and 15 [13]. In the course of this study, several patients were identified with unexpected alterations, affecting other loci than those on chromosome 11. These non-11p15 disturbances eluded routine diagnostic screening restricted to 11p15, but have significant impacts because they (a) help to define novel connections between imprinting disturbances and clinical features, and (b) increase the diagnostic yield [14].

As already mentioned, some IDs frequently show somatic mosaicism. This is particularly recognized for epimutations in SRS and BWS. The level of mosaicism shows a broad range, and can differ remarkably between different tissues [15, 16]. Therefore, if the tissue source of the diagnostic DNA sample has a level of mosaicism below the sensitivity of the diagnostic test, the result will be negative and the patient will escape diagnosis. The limited sensitivity of current single-locus tests therefore restricts diagnostic yield. Another limitation of the single-locus tests is that many of them do not differentiate different classes of mutations and epimutations associated with IDs (e.g. UPD, epimutation) (Figure 1, Table 2).

3.3 Limitation 3: ambiguous findings in IDs

Ambiguous clinical findings in IDs further challenge the value of current single-locus tests. The same clinical diagnosis may be associated with molecular alterations at different DMRs (e.g. ICR1 and ICR2 in 11p15 in BWS) and even at chromosomal loci (e.g. SRS: chromosomes 7 and 11; Table 1). This locus heterogeneity is further complicated by the possible occurrence of up to four different classes of mutations or epimutations. A prominent example is the recent discovery that *CDKN1C* gain-of-function mutations are associated with a clinical presentation very similar to SRS; this new aetiology complements the known causes of SRS clinical features, which include ICR1 hypomethylation, maternal UPD11 and 11p15 duplications [17, 18, 19, 20], not to mention maternal UPD7, and chromosomal aberrations.

The broadening of molecular testing shows that there is a considerable overlap between the different IDs, and the application of single-locus test can prevent the diagnosis of basic molecular defects and thus leave a patient without diagnosis. One example is the changing phenotype in the previously identified Temple syndrome (TS14), a congenital disorder linked

to imprinted loci on 14q32. Until recently, TS14 was regarded as a differential molecular diagnosis of Prader-Willi syndrome (PWS), but the broadening of genetic testing shows that the TS14 phenotype is heterogeneous, overlaps in early childhood with that of SRS, and is not mandatorily associated with (mild) psychomotoric retardation [21]. The impressive result of the application of multilocus tests in ID diagnostics is therefore obvious as a growing number of patients with Temple syndrome (TS14) can be identified among patients referred as SRS [22]. This illustrates the need for a comprehensive diagnostic algorithm in the testing of SRS. Furthermore, a considerable number of ID patients exhibit the above mentioned aberrant methylation at different imprinted loci (multilocus imprinting disturbances; MLID) [23] or carry at least two different molecular disturbances [24, 25] which escape single-locus testing. These patients often show a broad clinical spectrum and the phenotype may be ambiguous or even atypical for any known ID. In summary, as Table 1 shows, a similar molecular heterogeneity is known for the majority of IDs, and this heterogeneity is not captured by many of the available tests (Table 2).

5. Translational use of new techniques in IDs

An increasing number of new studies show that the aforementioned problems in diagnosis and investigation of IDs can be addressed by the use of new tests targeting multiple loci and/or a total exome and genome analysis.

One major prerequisite for the comprehensive diagnostic analysis of ID loci is the identification and definition of a standardized set of imprinted loci, DMRs and CpG islands. With the extensive characterization of imprinted methylation in molecularly normal and aberrant human tissues by a combination of whole-genome bisulfite sequencing and high-density methylation microarrays, Court and colleagues [26] recently laid the foundation for the development of harmonized tests. Another important step is the use of a controlled and standardized vocabulary for describing clinical entities, as is now provided by HPO (human phenotype ontology, http://www.human-phenotype-ontology.org/). To make the huge number of mutations and epimutations from different diagnostic and research institutions available to the public, the common use of LOVD (Leiden open variation database) as the common variation database is suggested (http://www.lovd.nl/3.0/home) (Figure 3).

The need for comprehensive testing to identify multiple and complex molecular alterations in ID patients and their clinical significance has been illustrated by numerous case reports and studies. For instance, in a patient with clinical features characteristic for Beckwith-Wiedemann syndrome (BWS; macrosomia, macroglossia, ear pits) and additional features (mental retardation, cardiac malformation, facial dysmorphisms), a paternal 11p15 duplication was identified by methylation-specific (MS) multiplex ligation-dependent probe amplification (MS-MLPA) [27]. Further characterization by SNP (single nucleotide polymorphism) array analysis and fluorescence in-situ hybridization (FISH) confirmed a 2.6 Mb duplication in 11p, but additionally identified a 4.9 Mb deletion in 18q22.3. Segregation analysis confirmed that the father and further family members were carriers of a balanced 11p/18q translocation. With the identification of the two different imbalances, the unusual clinical pattern in the patient could be explained, and the family could be precisely counseled. This case impressively illustrates the need and the power of the combined application of different tests. The exclusive use of the initial MS-MLPA restricted to 11p15 would have explained the BWS features, but not the other features, and particularly a lower recurrence risk would have been delineated for the family.

Another example for the impact of multilocus testing in IDs is the growing number of reports on BWS patients with a mosaic genome-wide paternal diploidy meaning that a significant ratio of cells carry chromosomes exclusively derived from the father. It turns out that a considerable number of BWS patients diagnosed to have an UPD(11p15)pat carry this unusual aberration [13], Considering that UPD(11p15)pat accounts for nearly 20% of BWS patients, and that carriers of a mosaic genome-wide paternal diploidy exhibit particularly unusual tumor histories which are not covered by the already existing surveillance programs for classical BWS [28], every case of UPD(11)pat warrants testing for genome-wide uniparental diploidy [29].

As already mentioned, somatic mosaicism is a common finding in IDs. In particular in the 11p15-associated disorders (BWS and SRS), nearly all patients with epimutations and UPD show mosaicism (for review: [13]). As a consequence of mosaicism, the molecular alterations currently often escape diagnostic detection in case of a low level mosaicism [30], an unequal distribution in different tissues [15], or an insufficient sensitivity of assays [31, 32]. New diagnostic approaches therefore may analyze different tissues from the same patient as well as apply multilocus and/or deep-sequencing tests.

The suitability of genome-wide SNP arrays for mosaicism detection has recently been demonstrated by Keren et al. [29]. Their data exhibited that SNP arrays are useful tools to estimate the sizes and mosaicism rates of UPD(11p15)pat as the basis for a more precise genotype phenotype correlation. The power to detect low-level mosaicism by array analysis was additionally confirmed by Prickett et al. [33] in a cohort of SRS patients: by hybridization of patients DNA onto DNA methylation microarrays the group provided proof of principle that this technique has a higher sensitivity than classical conventional single-locus tests. Additionally, the use of methylation arrays contributes to the idenfication of novel candidate imprinted genes, and the epigenomic profiling expands the understanding of normal methylome and its disruption [34].

For the same purposes, deep-sequencing NGS assays have been developed, and it has been shown that this technique is able to detect even low-level mosaicism [31, 32]. As it is generally observed for genetic testing, NGS will also improve the diagnostic workup in imprinting disorders, even in so far unexpected fields like non-invasive prenatal testing [35].

6. The new techniques contribute to the understanding of the pathoetiology of IDs

The aforementioned examples show that the application of multilocus and deep sequencing molecular tests are needed in ID diagnostics.

However, diagnostics and research should be regarded not as separate, but synergistic: the identification of new molecular alterations in ID patients enlighten the pathomechanisms in these heterogeneous disorders, while the data obtained from research strategies are translationally used for the improvement of diagnostic workups (Figure 3). This close relation between research and diagnostics will be illustrated with the following examples.

6.1 *ZFP57* mutations causing autosomal-recessively inherited IDs

In seven consanguineous families affected by the ID transient neonatal diabetes the genome-wide SNP array analysis delineated a single shared ~15Mb region of homozygosity on chromosome 6. Prioritization of candidate genes within this region prompted Sanger sequencing of the zinc-finger transcription factor *ZFP57*, which was shown to be mutated in all the consanguineous pedigrees [36]. *ZFP57* was independently shown in mouse to be essential for maintenance of imprinting marks in early development [37]. Moreover, in ethnicities with a high social rate of consanguineous union, *ZFP57* mutations may be the major cause of transient neonatal diabetes [38].

6.2 Families with *NLRP* mutations

Mutations of NLRP family genes *NLRP7* and *NLRP2* are associated with reproductive loss, where the females with these mutations gave birth to few or no liveborn children. Instead hydatidiform molar pregnancies with loss of maternal imprinting marks occurred. Caliebe et al. [39] reported exhaustive genetic and epigenetic analysis of a pedigree with two children with MLID. Exome sequencing identified a heterozygous missense variant in *NLRP7* in the mother of the children. Interestingly, she had inherited this variant from her mother and both genome-wide and targeted DNA methylation analysis showed that she, like her offspring, had MLID.

6.3 Identification of *CDKN1C* mutations as monogenetic causes of ID phenotypes

As with other genetic fields, deep sequencing NGS has significantly contributed to the understanding of IDs. Loss-of-function *CDKN1C* mutations are well known to be associated with BWS, but the first gain-of-function variants have been identified in patients with IMAGe syndrome (intrauterine growth restriction, metaphyseal dysplasia, adrenal hypoplasia congenita, genitourinary abnormalities)[40] and later in SRS [20]. This finding was remarkable in at least three different ways: (a) functionally, the opposite phenotypic outcomes of BWS vs. IMAGe/SRS could be explained by the opposite functional properties of the mutations (for review: [41]); (b) gain-of-function mutations cause growth restriction only in maternal inheritance, because *CDKN1C* is expressed only from the maternal allele, and therefore IMAGe syndrome can be regarded as an ID; (c) *CDKN1C* also illustrated the limitations of NGS. Due to its high GC content, the NGS coverage for *CDKN1C* was much lower than for other genomic regions, and was only identified after its sequence was reanalyzed with the Sanger method [40], and furthermore, the bioinformatics analysis had to be adapted to a pedigree model with an influence of the parent-of-origin of putative mutations.

6.4 NGS-based quantification of aberrant methylation contributes to the understanding of regulation of imprinting centers

As it could be recently shown by Beygo et al. [32], NGS-based approaches can also help to understand the functional interaction between different DMRs on the same imprinting center. One example is the chromosomal region 14q32, harboring the IG-DMR and the MEG3-DMR,.Genetic aberrations affecting these DMRs are associated with TS14 or Kagami-Ogata

Syndrome (KOS14, formerly known as paternal UPD14 syndrome). Based on the precise characterization of patients with small deletions in 14q32, affecting either the MEG3/DLK1 IG-DMR or the MEG3-DMR itself by quantitative next generation sequencing of bisulfite treated DNA samples, it could be shown that the two different DMRs have individual functional properties and a hierarchical order in that way that the IG-DMR is the dominant germline DMR and regulates the MEG3-DMR [32, 42].

7. Consequences of comprehensive analyses in IDs for their therapy

As already shown, the application of the new comprehensive and efficient laboratory tests in the diagnostic of IDs is resulting in an increase of patients with a molecular proven disorder. In particular the use of multilocus tests helps to identify patients with unexpected molecular alterations (e.g. with TS14-specific alterations in a cohort of SRS patients), to enlighten the molecular basis in case of unusual phenotypes [24] and to detect MLID carriers. In the latter group, it is currently discussed whether patients with MLID and a specific ID diagnosis have phenotypes different from those with “isolated” epimutations or mutations restricted to the disease-specific locus. Here further data are needed, however due to the first reports of monogenetic causes in MLID, genetic counselling might be different.
In general, the more precise determination of the molecular basis of a clinical picture is the basis for a more personalized treatment and management. One example is the recently reported evidence for different responses of SRS patients with different molecular disturbances on growth hormone treatment [43]. These findings can be regarded as a first step towards a more individual GH substitution. Another ideal example is the identification of genome-wide uniparental diploidy patients in the group of BWS patients: these patients' tumor history and risks are different from those of UPD11pat alone, and therefore require another tumor surveillance program [28].

Potentially the new therapeutic options for IDs which can be delineated from the rapid development of new methods are more promising. With the exception of those patients with chromosomal aberrations or gene mutations, carriers of epimutations can have a normal genome. This means these patients carry intact, but silenced alleles. Thus, these defects should in principle be curable by reversing the aberrant imprinting mark. With the growing knowledge on the molecular basis of aberrant imprinting from experimental models and the development of powerful strategies towards cell replacement therapies, e.g. induced

pluripotent stem cells (iPS) and the gene editing CRISPR/Cas system, promising milestones towards a causative therapy have been undertaken [44, 45, 46].

Conclusions

The rapid development of efficient and high-throughput molecular techniques and their application in research and diagnostics in the last decade have led to an impressive increase of knowledge on IDs and their basic pathomechanisms. In combination with the improvement of data recording and documentation, the diagnostic strategies are increasingly based on standardized protocols, and thereby provide the backbone for a directed counselling as well as of a more personalized conventional and new therapeutic approach.

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Tables and Figures

Table 1: The known congenital disorders associated with disturbances at imprinted loci, their frequencies, and the associated molecular and clinical findings. (NR not yet reported, IUGR intrauterine growth retardation, PNGR postnatal growth retardation, PTH parathormone; hypom. hypomethylation; hyperm. hypermethylation)(*absolute numbers for the frequencies of the molecular subtypes are taken from representative studies or reviews; ** in case of AS and PWS these frequencies are well established from huge cohorts and therefore not documented by specific references)

Table 2: Methylation-specific (MS) assays applied in diagnostics and research of imprinted loci.

Figure 1: Four different molecular mechanisms affecting imprinted regions can be detected in IDs, they all result in a disturbed expression of imprinted genes.

Figure 2: Physical localization of the target CpGs in 11p15.5 of the different molecular tests applied by the contributing groups. The CpGs detected by the MS-MLPA assay are marked by asterisks, the CpG stretches covered by the other tests are shown as bars. (*On the Y axis, the methylation is indicated, for imprinted loci an averaged methylation of 50% can be observed.)

Figure 3: Application of standardized and harmonized methods for recording, documentation and diagnostic as the basis for research and personalized medicine. These exchanges and interactions are main topics of the European network of congenital imprinting disorders (www.imprinting-disorders.eu).

Table 1

Disorder	Chromosomal region	Molecular Disturbance	Frequencies (n=)	Multilocus Defects	Clinical features
Transient Neonatal Diabetes mellitus (TNDM)	6q24	UPD(6)pat dup(6q) *PLAGL1* hypom. *ZFP57* mutations	41% 29% 30% 50% (n=163) [34]	50%	IUGR, transient diabetes, hyperglycemia without ketoacidosis, macroglossia, omphalocele
Silver-Russell syndrome (SRS)	7	UPD(7)mat	7-10% (n=109)[13]	1 case	IUGR/PNGR, rel. macrocephaly, hemihypotrophy, triangular face, feeding difficulties
	11p15.5	UPD(11)mat dup(11p15)mat ICR1 hyp. *CDKN1C* mutations *IGF2* mutations	n=1 1-2% >38% n=1 n=1 (n=109)[13]	- - ~10% - -	
Beckwith-Wiedemann syndrome (BWS)		UPD(11)pat Genomewide paternal UPD dup(11p15)pat ICR1 hyperm. ICR2 hypom. *CDKN1C* mutations	20% ~ 10%? ~ 90% 1-2% 4% 50% 5% [n=40)[13]	- - - 25% -	pre- and postnatal overgrowth, organomegaly, macroglossia, omphalocele, neonatal hypoglycemia, hemihypertrophy, increased tumour risk
Temple syndrome (UPD(14)mat)	14q32	UPD(14)mat del(14q32)pat *MEG3* hypom.	78.4% 9.8% 11.7% (n=51)[21]	- - NR	IUGR,PNGR, Hypotonia, feeding difficulties in infancy, truncal obesity, scoliosis, precocious puberty
Kagami-Ogata syndrome (UPD(14)pat)		UPD(14)pat del(14q32)mat MEG3 hyperm.	65.4% 19.2% 15.4%	- - NR	IUGR, polyhydramnion, abdominal and thoracal wall defects, bell-shaped thorax, coat-hanger ribs

			(n=34)[42]		
Angelman syndrome (AS)	15q11q13	UPD(15)pat del(15q11q13)mat aberrant methyl. *UBE3A* mutations	1-2% 75% ~3% 5-10%**	- - - -	mental retardation, microcephaly, no speech, unmotivated laughing, ataxia, seizures
Prader-Willi syndrome (PWS)		UPD(15)mat del(15q 11q13)pat aberrant methyl.	25-30% 70-75% ~1%**	- - 1 case	PNGR, mental retardation, neonatal hypotonia, hypogenitalism, hypopigmentation, obesity/ hyperphagia
Precocious puberty	15q	MKRN3 mutations	Unknown	5 families	Precocious puberty (girls: 5.75 years, boys: 8.10 years)
Pseudohyperparathyreoidism	20q13	UPD(20)pat; aberrant methyl.	Unknown	- 12.5%	Resistance to PTH and other hormones; Albright hereditary osteodystrophy; Subcutaneous ossifications Feeding behaviour anomalies; Abnormal growth
UPD(20)mat	20	UPD(20)mat	Unknown	9 cases	IUGR, PNGR, failure to thrive

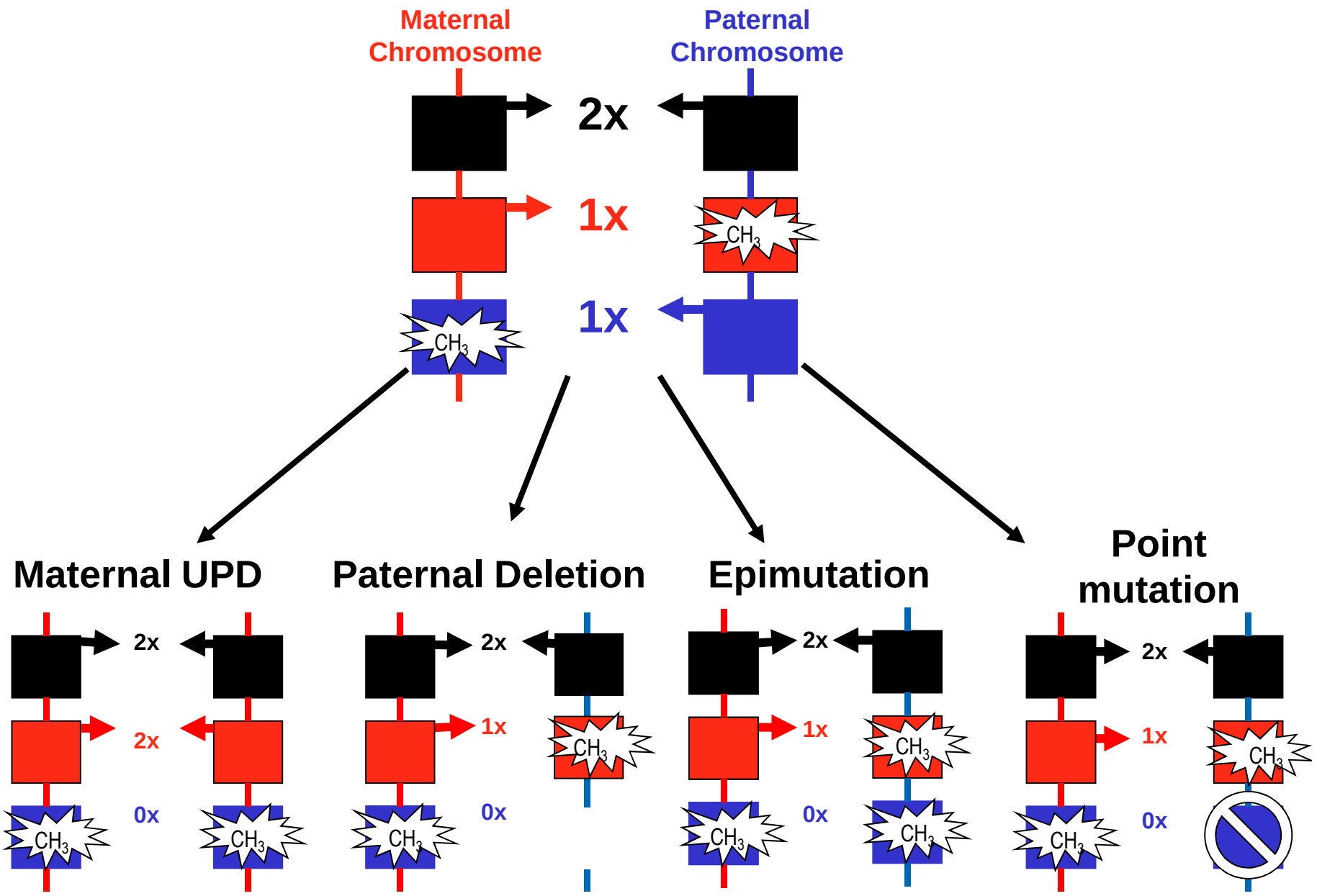
Table 2

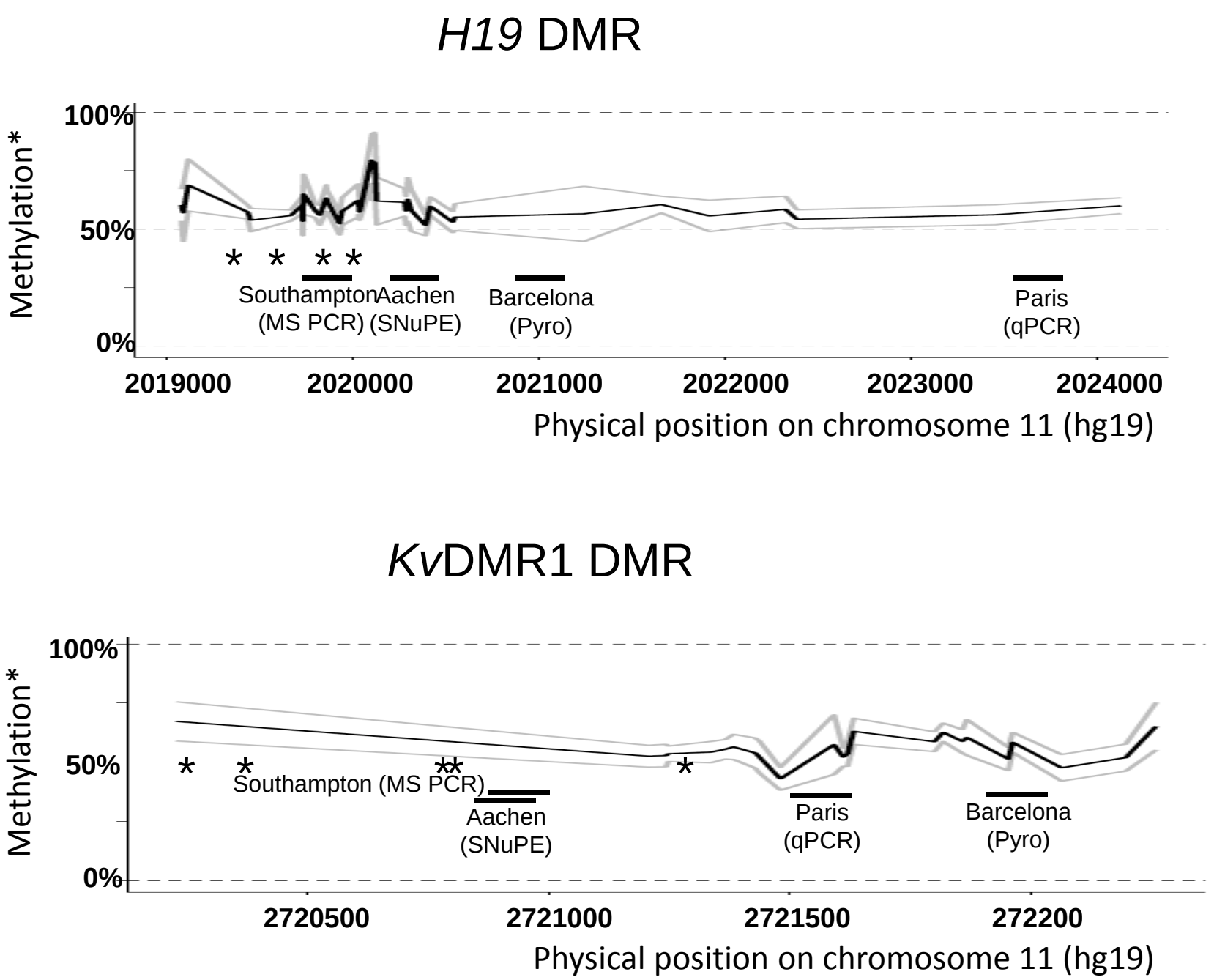
Method	Description	Loci per test	Detection of				Advantages	Disadvantages
			UPD	Epimutation	CNV	SNV		
Single locus tests								
MS Southern Blot	Classical Southern-Blot analysis using methylation-sensitive restriction endonucleases to differentiate between methylated and unmethylated alleles	1	Y	Y	Y	N	Semi-quantitative	Large amounts of DNA, time-consuming, no discrimination between the different types of (epi)mutations, low sensitivity
Bisulfite sequencing	Primary amplification of bisulphite-treated DNA by primers not discriminatory for methylation status of amplicon, followed by cloning of amplicons, and sequencing of bisulfite-altered differentially methylated cytosines within individual clones	1*	Y	Y	N	N	Semi-quantitative (amplification bias!)	Time consuming, costly, cloning needed, no discrimination between the different types of (epi)mutations
MS PCR	Single-tube assay amplifying bisulfite-treated DNA with one common labelled primer and two primers specific to methylated or unmethylated sequence, giving differently-sized products distinguished by genotyping	1	Y	Y	Y	N	Fast, cheap, semi-quantitative	no discrimination between the different types of (epi)mutations
QAMA real-time PCR-based	qPCR assay to differentiate between methylated and unmethylated alleles by the	1	Y	Y	Y	N	Fast, quantitative	no discrimination between the different types of

methylation assay	use of differentially labeled probes							(epi)mutations
Microsatellite analysis (STR)	Amplification of microsatellite repeat sequences followed by resolution of amplicon sizes to determine inheritance pattern of alleles	1	Y	N	Y	N	Fast, cheap, quantification possible	DNA of at least one parent required; no discrimination between the different types of (epi)mutations; microsatellites only in genomic vicinity of DMRs
MS HRM	Primary amplification of bisulphite-treated DNA by primers not discriminatory for methylation status of amplicon, followed by high-resolution DNA melting analysis to distinguish melting characteristics of differentially methylated alleles	1	Y	Y	Y	N	Fast, cheap, semi-quantitative	no discrimination between the types of (epi)mutations
Multi-Locus tests								
MS pyrosequencing	Primary amplification of bisulphite-treated DNA by primers not discriminatory for methylation status of amplicon, followed by sequence detection of bisulfite-altered differentially methylated cytosines within amplicon	several	Y	Y	Y	N	Fast, quantitative	no discrimination between the types of (epi)mutations
MS-MLPA (Methylation-specific multiplex ligation-dependent probe amplification)	locus-specific MLPA probe-pairs hybridize to DNA. A successful binding of both probes allows their ligation and subsequent amplification. The parallel use of a methylation-sensitive restriction enzyme allows DNA quantification and determination of the methylation status	Up to ~46	Y	Y	Y	N	Ready to use kits. Free and easy to use analysis software. Direct discrimination of CNVs and Epimutations.	Mostly bound to already available kits Relatively high DNA Quality needed.

MS-SNuPE (Methylation-specific single nucleotide primer extension)	based on the ABI PRISM SNaPshot Multiplex kit using bisulfite converted DNA and specific SNuPE-Primers. The primers are designed to hybridize directly in front of a known CpG. The use of ddNTPs in the sequencing reaction allows a single-base difference depending on the methylation status of the amplified allele.	Up to 10	Y	Y	Y	N	Flexible and adjustable use of SNuPE Primers. Cheap	Primers not commercially available. Mostly self-made analysis software. Only indirect discrimination of CNVs and Epimutations.
Molecular Karyotyping (arrayCGH, SNP array)	Cohybridization of genomic DNA to complementary DNA fragments or hybridization to oligonucleotides spotted on an array surface. Resolution depends on the number of spotted DNA fragments and their distribution. Two main techniques are available: Array CGH -Comparative genomic hybridization: comparison of a test sample and reference sample allows the identification of relative copy number changes. SNP Array: test samples are compared in-silico to a reference genome. Spotted DNA-Fragments include SNPs	Whole genome: depends on resolution	Y (SNP) N (CGH)	N	Y	N	Fast technique to obtain whole genome CNV information. Relatively cheap Detection of low-level mosaicism	Only unbalanced alterations are detected. Does not detect Epimutations, and in case of arrayCGH, not UPDs
MS-Array, e.g. Infinium 450K	With a comparable approach to the conventional molecular Array technology methylation specific Arrays use bisulfite converted DNA for analysis which can hybridize to genomewide CpG sites spotted to the Arrays surface.	~ 475k CpGs, of which 100s in DMRs of classically	Y	Y	Y	N	Genomewide CpG coverage	Expensive Relatively low resolution (only covers CpGs) Not all differentially methylated regions are represented on the array,

		imprinted genes						though coverage of most is excellent
NGS / RRBS / WGBS	Next generation sequencing (NGS)/RRBS (reduced representation bisulphate sequencing)/WGBS (whole genome bisulphate sequencing) describes massive parallel non-Sanger-based high-throughput DNA sequencing technologies of either genomic or bisulfite treated DNA.	Assay dependent : up to whole genome	Y	Y	Y	Y	Whole genome / epigenome can be analysed at once Price per base relatively low Detection of low-level mosaicism	Repetitive regions/ CG rich regions have an impact on coverage and therefore might hinder the analysis Large data challenges requiring advanced bioinformatics infrastructure and knowledge. Incomplete bisulphite modification has to be considered
MeDIP	Methylated DNA immunoprecipitation (MeDIP or mDIP) uses antibodies against 5-methylcytosine (5mC) to enrich methylated DNA sequences in a genome wide or chromosome wide scale. The enriched methylated DNA can be used for downstream high throughput applications like NGS MeDIPseq or array based methods (MeDIP-chip)	Assay dependent : up to whole genome	Y	Y	(Y)	N	Genomewide CpG coverage on methylated regions	Cross reactivity of antibodies, no single CpG resolution




H19 DMR
Methylation*
100%
50%
0%
Southampton (MS PCR)
Aachen (SNuPE)
Barcelona (Pyro)
Paris (qPCR)
2019000
2020000
2021000
2022000
2023000
2024000
Physical position on chromosome 11 (hg19)
KvDMR1 DMR
Methylation*
100%
50%
0%
Southampton (MS PCR)
Aachen (SNuPE)
Paris (qPCR)
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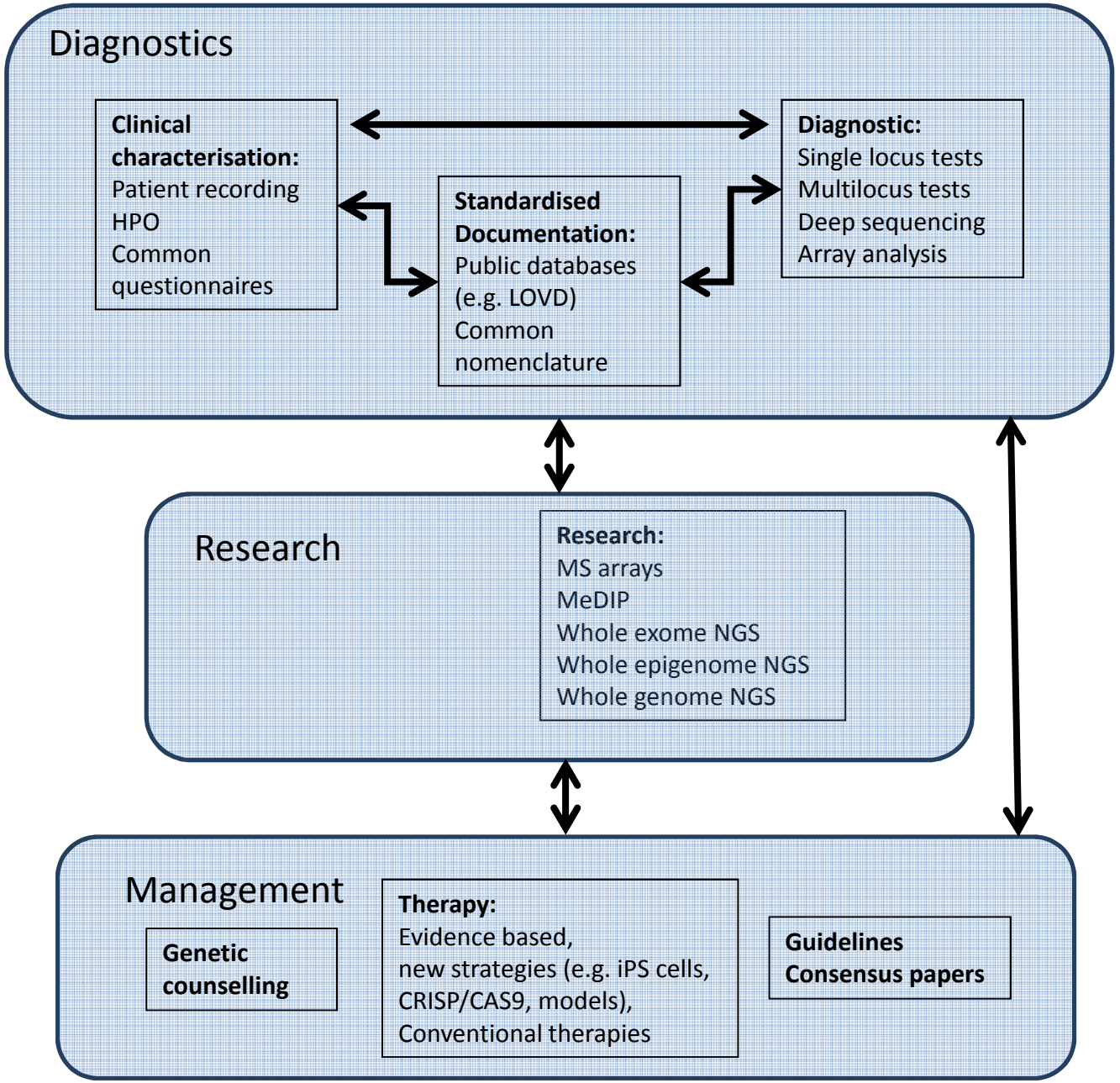
Physical position on chromosome 11 (hg19)



Highlights

- Imprinting disorders (IDs) are a group of congenital diseases affecting growth, development and metabolism.
- IDs are caused by changes in the allele-specific regulation ("epigenetic mutation") or in the genomic sequence ("genetic mutation") of imprinted genes.
- The application of single locus tests restrict diagnostic yield.
- A standardization in testing methodology, in the DNA sequences tested, and in clinical inclusion criteria prompting testing is urgently needed.
- The application of efficient and hight-throughput molecular techniques lead to an impressive increase of knowledge on IDs and their basic pathomechanisms.
- The diagnostic strategies will be based on standardized protocols, and provide the backbone for directed counselling, more personalized management, and new therapeutic approaches.