Molecular Basis and Diagnostic Approach to Isolated and Syndromic Lateralized Overgrowth in Childhood

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Objective To demonstrate a high-yield molecular diagnostic workflow for lateralized overgrowth (LO), a congenital condition with abnormal enlargement of body parts, and to classify it by molecular genetics.

Study design We categorized 186 retrospective cases of LO diagnosed between 2003 and 2023 into suspected Beckwith-Wiedemann spectrum, PIK3CA-related overgrowth spectrum (PROS), vascular overgrowth, or isolated LO, based on initial clinical assessments, to determine the appropriate first-tier molecular tests and tissue for analysis. Patients underwent testing for 11p15 epigenetic abnormalities or somatic variants in genes related to PI3K/AKT/mTOR, vascular proliferation, and RAS-MAPK cascades using blood or skin DNA. For cases with negative initial tests, a sequential cascade molecular approach was employed to improve diagnostic yield.

Results This approach led to a molecular diagnosis in 54% of cases, 89% of cases consistent with initial clinical suspicions, and 11% reclassified. Beckwith-Wiedemann spectrum was the most common cause, with 43% of cases exhibiting 11p15 abnormalities. PIK3CA-related overgrowth spectrum had the highest confirmation rate, with 74% of clinically diagnosed patients showing a *PIK3CA* variant. Vascular overgrowth demonstrated significant clinical overlap with other syndromes. A molecular diagnosis of isolated LO proved challenging, with only 21% of cases classifiable into a specific condition.

Conclusions LO is underdiagnosed from a molecular viewpoint and to date has had no diagnostic guidelines, which is crucial for addressing potential cancer predisposition, enabling precision medicine treatments, and guiding management. This study sheds light on the molecular etiology of LO, highlighting the importance of a tailored diagnostic approach and of selecting appropriate testing to achieve the highest diagnostic yield. *(J Pediatr 2024;274:114177)*.

Lateralized overgrowth (LO), also known as segmental overgrowth, is defined as an increase in the size of any region of the body compared with normal.[1] LO results in body asymmetry, such as an increase in the length or girth of a limb compared with the contralateral one, and is considered pathological if exceeding the contralateral by ≥10%.[2] Despite its potential implications for prognosis, follow-up, and screening for cancer predisposition, LO is often underdiagnosed, and its molecular etiology is frequently underinvestigated.[3] LO can be caused by somatic genetic or epigenetic defects that result in excessive cell proliferation and survival, leading to tissue overgrowth. It can be isolated—that is, not accompanied by other features and referred to as isolated LO (ILO)—or a core feature of several overgrowth syndromes.[4] The most common overgrowth syndromes presenting with LO include Beckwith-Wiedemann spectrum (BWSp), PIK3CA-related overgrowth spectrum (PROS), and somatic pathogenic variants in genes of the PI3K/AKT/mTOR pathway other than *PIK3CA*.

BWSp	Beckwith-Wiedemann spectrum
HD-NGS	High-depth next-generation sequencing
IC1-GoM	Imprinting center 1 gain-of-methylation
IC1-LoM	Imprinting center 1 loss-of-methylation
IC2-LoM	IC2 loss-of-methylation
ILO	Isolated overgrowth
LO	Lateralized overgrowth
MS-MLPA	Mathylation-sensitive multiple ligation probe analysis
PROS	PIK3CA-related overgrowth spectrum
SRS	Silver Russell syndrom
UPD(11)pat	Paternal uniparental disomy of chromosome 11

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1. BWSp us characterized by several anomalies and tumor predisposition, caused by epigenetic mosaic anomalies of 2 gene clusters involved in cell growth in the 11p15.5 chromosomal region, as Imprinting Center 1 Gain-of-Methylation, IC2 Loss-of-Methylation (IC2-LoM), and paternal uniparental disomy of chromosome 11 (UPD(11)pat).[5,6]
2. PROS is caused by somatic gain-of-function variants in *PIK3CA* leading to oversignaling through the PI3C/AKT/mTOR cellular cascade and cell proliferation.[7] PROS encompass several phenotypes defined by the affected body region and the type of tissue with overgrowth and is often accompanied by recognizable signs (vascular anomalies, skin naevi, fat deposition, and dysmorphisms).[8-10]
3. Somatic pathogenic variants in genes of the PI3K/AKT/mTOR pathway other than *PIK3CA* (including *AKT3*, *PIK3R1*, *MTOR*, *TEK*, and *PTEN*), *GNA11*, *GNAQ*, or of the RAS-MAPK pathway (*RASA1 KRAS*, *NRAS*, *PTPN11*, and *NF1*).[11-19]

Although specific guidance and follow-up strategies are available for many of these conditions, difficulties arise when LO is the main presentation and other features are lacking or attenuated. ILO can represent the milder end of any of these syndromes. Molecular diagnosis in ILO patients allows for the reclassification of the disease in the appropriate molecular spectrum, with the corresponding clinical implications, making a precise definition of the underlying molecular bases crucial. For instance, LO is typically self-limiting during growth in BWSp, but require specific cancer screening differentiated based on the epigenotype.[2,4,20] In contrast, PROS carries risk of severe and lifelong progression of LO, has almost no cancer implications, and can be modulated by PIK3CA-inhibitors.[2,21-23] Identifying the underpinning molecular anomaly in a patient may pave the way to targeted therapies with a precision medicine approach. For example, MEK inhibitors have shown promising results in several mosaic RASopathies, sirolimus has been used in many non-BWSp LO, and alpelisib (BYL719) is currently undergoing phase 2 clinical trial in PROS patients recently receiving accelerated approval by the Food and Drug Administration for this indication in the US.[24-27]

The molecular diagnostics of LO are complex and often challenging, because its many biological derangements are usually somatic and mostly not detectable in blood-extracted DNA: LO often require molecular studies on tissue-extracted DNA and technologies able to detect low-frequency mosaicisms. The recent introduction of such technologies has allowed classification of LO according to the deregulated cellular pathway. However, despite these advances, epidemiological data remain limited and a standardized diagnostic approach is still lacking.[28,29] This retrospective study of 186 patients with LO provides epidemiological data on its molecular etiology and demonstrates a high-yield diagnostic workflow, providing patients and clinicians with improved follow-up strategies, more treatment options, and refinement in screening procedures.

Methods

Study Design and Patients

Data were collected retrospectively from patients followed for LO at the Pediatric Clinical Genetic Unit of the Regina Margherita Children's Hospital in Turin, Italy from 2003 to 2023. This study was approved by the local ethics committee (Institutional Review Board 86/2022 #35286, March 2022).

The recorded data included age, sex, clinical information on LO features (including affected body areas, measurements based on the type of overgrowth, imaging studies, and photographic documentation), the clinical diagnosis/suspect at the first examination, the molecular analysis performed, and the final diagnosis.

Patients with LO were classified into 4 categories based on the initial clinical diagnosis.

a. BWS spectrum (ie, with LO and ≥1 BWSp criteria).[5]
b. PROS spectrum (LO and ≥1 PROS diagnostic criteria.[10]
c. Vascular overgrowth (VO), defined as isolated vascular anomaly of an overgrown body area resulting from a vascular anomaly, and no features suggestive of other syndromic LO.[17,30,31]
d. ILO, that is, with isolated overgrowth and no other features suggestive of any the abovementioned disorders.

Diagnostic Flowchart for Molecular Testing

Patients underwent different diagnostic management depending on their initial suspected diagnosis (**Figure 1**).

Those within the BWSp group underwent a methylation-sensitive multiple ligation probe Analysis (MS-MLPA) for the imprinted 11p15.5 chromosomal region on peripheral blood leukocytes DNA.[32] For those negative on that initial test, MS-MLPA on DNA extracted from skin of the LO area by a punch biopsy was proposed. In case the latter proved negative, DNA from biopsy was further evaluated for variants in genes of the PI3K/AKT/mTOR cascade, associated with vascular proliferation or RAS-MAPK pathways by high-depth next-generation sequencing (HD-NGS) as long as sufficient DNA was available. Patients with suspected PROS underwent analysis of DNA extracted from the skin of the overgrown area, using a custom HD-NGS panel for of the genes of the PI3K/AKT/mTOR pathway. For those with suspected central nervous system involvement (such as megalencephaly-capillary malformation or [di])-hemi-megalencephaly) DNA extracted from a buccal swab was tested before DNA extracted from skin biopsy.[33] If no variants in genes of the PI3K/AKT/mTOR were found, genes of the vascular proliferation and RAS-MAPK pathways were tested and, if negative, MS-MLPA for the 11p15.5 chromosomal region was conducted sequentially as long as enough DNA was available.

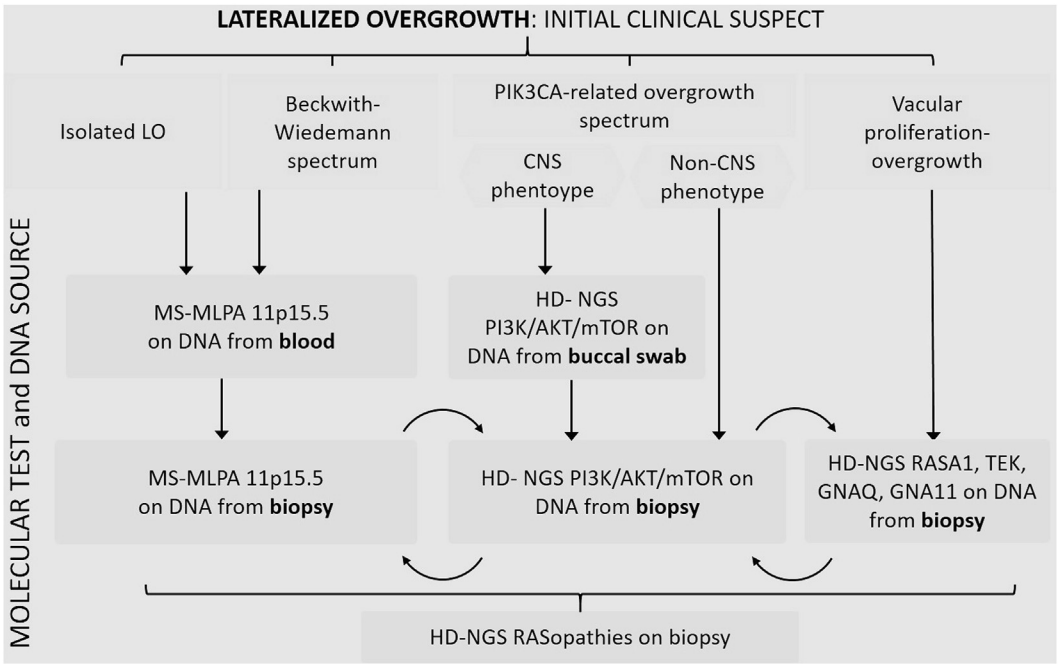

Figure 1. Workflow of the diagnostic management depending on the initial diagnostic suspicion, including the type of molecular test and source of DNA used. Patients with LO were classified in 4 groups: BWSp,[5] PROS,[10] VO, or ILO. Patients were then tested with different molecular approach based on the initial clinical suspect.

Patients with suspected VO underwent cascade analysis on DNA extracted from a biopsy of the vascular malformation, beginning with a panel including the additional vascular malformations genes *TEK*, *GNAQ*, *GNA11*, and *RASA1* and, if negative, further tested with a third panel including additional genes of the RAS-MAPK pathways, as long as sufficient DNA was available. Patients with ILO underwent the same procedure as those affected suspected with BWSp (see a).

Skin biopsy was proposed to patients only when deemed clinically beneficial in term of the benefit to harm ratio. The procedure was discussed with the parents, highlighting the expected improvement in the diagnostic yield, the clinical relevance of obtaining a precise molecular diagnosis, and the potential benefits for follow-up.

Biological Samples and Molecular Analyses

Written informed consent for DNA testing and tissue biopsies was obtained prior to any procedures. Sample management was different based on tissue tested. DNA was extracted either from peripheral blood mononucleate cells, affected skin or vascular malformation tissue (n = 53) or cultured fibroblasts (n = 28), or buccal swab. Blood was preserved in EDTA tubes. Oral swab and tissues were preserved in physiologic saline into a sterile test tube. All samples were preserved at +4 °C until DNA extraction (within 48 hours from sampling). Genomic DNA was extracted from skin sample of the visible vascular markings using the QIAamp Mini Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions and was quantified on a BioSpectrometer Plus (Eppendorf, Hamburg, Germany).

Molecular analyses for 11p15.5 abnormalities were carried out by MS-MLPA test kit SALSA ME030 (MRC Holland, Amsterdam, the Netherlands). The protocol used is available at www.mrc-holland.com. The amplified product was sequenced through capillary electrophoreses on the 3500xL Dx Genetic Analyzer (Life Technologies, Carlsbad, CA) and data were processed with the Coffalyser software v.220513.1739 (MRC Holland). MS-MLPA allows to detect in a single experiment IC2-LoM, imprinting center 1 gain-of-methylation (IC1-GoM), imprinting center 1 loss-of-methylation (IC1-LoM), and UPD(11)pat. The latter was confirmed by either high-resolution polymorphism or microsatellite analysis.[34] *CDKN1C* analysis was performed in 7 patients (all negative) and not routinely, based on accepted recommendations, because *CDKN1C* variants are germline in BWSp, so usually do not manifest with LO as a phenotypic feature.[21,35]

For testing somatic variants, 3 different custom NGS panels were used including, respectively, 21 genes (*PIK3R1*, *PIK3R2*, *PIK3CA*, *PTEN*, *PDK1*, *PDK2*, *KRAS*, *AKT1*, *AKT2*, *AKT3*, *RICTOR*, *MAPKAP1*, *MLST8*, *MTOR*, *IRS1*, *GAB1*, *GAB2*, *THEM4*, *MAPK8I1*, *PTPN11*, and *RAPTOR*), 9 genes (*RASA1*, *TEK*, *TSC2*, *GNAQ*, *TSC1*, *DEPDC5*, *CCND2*, *NPRL3*, and *GNA11*), and 15 genes (*A2ML1*, *BRAF*, *CBL*, *HRAS*, *KRAS*, *MAP2K1*, *MAP2K2*, *NF1*, *NRAS*, *PTPN11*, *RAF1*, *RT1*, *SHOC2*, *SOS1*, and *SPRED1*). After 2021, the genes of the first and the second NGS panels were merged into a single custom panel, including *PIK3CA*, *PIK3R1*, *PIK3R2*, *TEK*, *TSC2*, *GNAQ*, *TSC1*, *MTOR*, *PTEN*, *AKT3*, *AKT2*, *DEPDC5*, *AKT1*, *CCND2*, *NPRL3*, *GNA11*,

Table. Final diagnosis of the cases with conclusive molecular tests including type of molecular defect, variant classification, and variant allele frequency

Initial clinical suspects	Molecular abnormality and pathogenic variants found	Final diagnosis
BWSp (n = 79)	IC1-GoM (n = 4)	BWSp
	IC2-LoM (n = 24)	
	UPD(11)pat (n = 15)	
ILO (n = 56)	IC1-GoM (n = 1)	BWSp
	IC2-LoM (n = 1)	
	UPD(11)pat (n = 8)	
	IC1-LoM (n = 1)	Silver-Russel syndrome
	PIK3CA:c.263G>A; p.Arg88Gln (pathogenic, 34%)	PROS
	PIK3CA:c.263G>A; p.Arg88Gln (pathogenic, 27%)	
PROS (n = 39)	*PIK3CA*:c.323G>A; p.Arg108His (likely pathogenic, 6%)	PROS
	PIK3CA:c.344G>C; p.Arg115Pro (likely pathogenic, 4.6%)	
	PIK3CA:c.1132T>C; p.Cys378Arg (pathogenic, 7%)	
	PIK3CA:c.1133G>A; p.Cys378Tyr (pathogenic, 26%)	
	PIK3CA:c.1133G>A; p.Cys378Tyr (pathogenic, 37%)	
	PIK3CA:c.1136C>A; p.Cys378Tyr (likely pathogenic, 4%)	
	PIK3CA:c.1356_1358delAGA; p.Glu453del (pathogenic, germline)	
	PIK3CA:c.1357 G > A; p.Glu453Lys (pathogenic, 3%)	
	PIK3CA:c.1624G>A; p.Glu542Lys (pathogenic, 4%)	
	PIK3CA:c.1633G>A; p.Glu545Lys (pathogenic, 3%)	
	PIK3CA:c.1636C>A; p.Glu546Lys (likely pathogenic, 5%)	
	PIK3CA:c.2176G>A; p.Glu726Lys (pathogenic, 9.4%)	
	PIK3CA:c.2176G>A; p.Glu726Lys (pathogenic, 8.3%)	
	PIK3CA:c.2176G>A; p.Glu726Lys (pathogenic, 13%)	
	PIK3CA:c.2740G>A; p.Gly914Arg (pathogenic, 8%)	
	PIK3CA:c.3073A>G; p.Thr1025Ala (pathogenic, 11%)	
	PIK3CA:c.3074C>A; p.Thr1025Asn (likely pathogenic, 6%)	
	PIK3CA:c.3104C>T; p.Ala135Val (pathogenic, n.a.)	
	PIK3CA:c.3127A>G; p.Met1043Val (likely pathogenic, 2%)	
	PIK3CA:c.3129G>A; p.Met1043Ile (pathogenic, 7%)	
	PIK3CA:c.3139C>T; p.His194Tyr (pathogenic, 15%)	
	PIK3CA:c.3140A>G; p.His1047Arg (pathogenic, 9%)	
	PIK3CA:c.3140A>G; p.His1047Arg (pathogenic, 15%)	
	PIK3CA:c.3140A>G; p.His1047Arg (pathogenic, 7%)	
	PIK3CA c.3140A>G; p.His1047Arg (pathogenic, 3%)	
	PIK3CA:c.3140A>G; p.His1047Arg (pathogenic, 7%)	
	PIK3CA c.3140A>G; p.His1047Arg (pathogenic, 11%)	
	PIK3CA:c.3140A>G; p.His1047Arg (pathogenic, 9%)	
	PIK3CA:c.3140A>G; p.His1047Arg (pathogenic, 3.2%)	
	mTOR:c.4448G>A; p.Cys1283Tyr (pathogenic, 32%)	Other genes of the PI3K/AKT/mTOR pathway
	AKT3:c241_243dup; p.Thr81dup (likely pathogenic, 8%)	
	GNA11:c.547C>T; p.Arg183Cys (pathogenic, 3%)	VO
	GNAQ: c.548G>A; p.Arg183Gln (pathogenic, 3%)	
	GNAQ: c.548G>A; p.Arg183Gln (pathogenic, 4%)	
VO (n = 12)	*GNA11*:c.547C>T; p.Arg183Cys (pathogenic, 2.8%)	VO
	GNAQ:c.548G>A; p.Arg183Gln (pathogenic, 4%)	
	RASA1:c.768C>A; p.Tyr256Ter (pathogenic, germline)*	
	RASA1:c.1253+1G>A; p.? (likely pathogenic, germline)	
	TEK:c.2740C>T; p.Leu914Phe (likely pathogenic, 6.4%)	
	TEK:c.2740C>T; p.Leu914Phe (pathogenic, 8%)	
	PIK3CA:c.2740G>A; p.Gly914Arg (pathogenic, 24%)	PROS
	PIK3CA:c.1412C>T; p.Pro471Leu (likely pathogenic, 6%)	
	PTPN11:c.1520C>A; p.Thr507Lys (pathogenic, 12%)	Mosaic RASopathy
	2 × *PTEN*:c491delGTT; p.Lys164ArgfsTer3 (likely pathogenic, germline)*	PTHS

PTHS, PTEN-hamartoma tumor syndrome.
*Familial case.

and *RASA1* genes. The custom panels were designed online using the Design Studio tool supplied by Illumina (http://designstudio.illumina.com) and the libraries were prepared with AmpliSeq Illumina Kit (Illumina, San Diego, CA) according to the manufacturer's instructions. Massive sequencing of amplicons was carried out on the exonic regions and on the intronic regions adjacent (±25 bp) to the genes. Sequencing runs were performed on Illumina MiSeq instrument according to the manufacturer's instructions with a uniformity of base coverage of 99,.5%. The reading depth was >2000 reads per base. Data analysis was performed by Local Run Manager (Illumina) and BaseSpace Variant Interpreter software (Illumina) and clinical significance was defined using online databases, including dbSNP (www.ncbi.nlm.nih.gov/snp), gnomAD (gnomad.broadinstitute.org), and ClinVar (www.ncbi.nlm.nih.gov/clinvar). Variants were classified according to American College of Medical Genetics guidelines. Eligible variants were confirmed by Sanger

sequencing on SeqStudio when a sufficient variant allele frequency was detected. Samples were submitted for somatic variant testing to the laboratory of the Department of Precision and Regenerative Medicine and Ionian Area, Medical Genetics Section of University Hospital Consortium Corporation Polyclinics of Bari.

Results

A total of 186 patients with LO were included in this study, comprising 95 females and 91 males, with a median age of 11.8 ± 12.9 years. All patients were children (<18 years) at their first evaluation, with 40 of them being adults by the end of the follow-up period. Among the 186 patients, 79 had suspected BWSp (42.5%), 39 suspected PROS (21.0%), 12 suspected VO (6.4%), and 56 ILO (30.1%). All patients underwent ≥1 molecular test: 105 only on blood DNA and 81 on DNA from diagnostic skin biopsy, of which 30 tested on blood before and 51 beginning directly from biopsy. In 101 patients (54.3%), a molecular diagnosis was made: 49 (48.5%) based on blood-extracted DNA and 52 (51.5%) on tissue-extracted DNA. In 90 cases (89.1%), the molecular diagnosis was consistent with the initial clinical suspicion, whereas in 11 (10.9%) the molecular diagnosis was different from that clinically suspected (**Table**). **Figure 2** summarizes the flow of molecular tests performed based on initial suspicion, and **Figure 3** shows the diagnoses for each molecular test performed in each of the conditions initially suspected.

BWSp

Among the 79 patients suspected to have BWSp, 43 (54.4%) tested positive for 11p15.5 methylation abnormalities on peripheral blood DNA: 24 had IC2-LoM, 15 UPD(11)pat, and 4 IC1-GoM. Diagnostic skin biopsy was offered to 17 of the 36 negatives, because it was considered potentially helpful in terms of benefit and harm to test further tissue: most patients >8 years of age with a clinical diagnosis of BWSp (ie, a score of ≥4) did not undergo a biopsy as they were out of their cancer screening window. Nine patients declined to undergo the procedure. Of the remaining 8 cases who underwent a biopsy, 2 failed DNA extraction and 6 were submitted to MS-MLPA on tissue-extracted DNA: 2 (33.3%) had a UPD(11)pat and 4 were negative. The latter underwent cascade NGS tests for somatic variants, but none were diagnosed with a specific disease. Of the 36 (45.6%) who tested negative initially, none were reclassified with other LOs.

PROS

Of the 39 patients with suspected PROS, 12 (30%) had central nervous system involvement. Among these, 11 (27.5%) were tested for somatic variants on DNA from buccal swabs, and 6 were found to have a pathogenic *PIK3CA* variant (54.5%). NGS analysis of the PI3K/AKT/mTOR pathway genes was performed on DNA from affected skin punch biopsy in 34 patients. This includes the 5 patients who tested negative on buccal swab-extracted DNA, 1 patient with megalencephaly-capillary malformation who did not undergo buccal swab testing, and the 28 patients with non-central nervous system phenotypes. Of the 34 patients, 25 (73.5%) had a pathogenic variant identified, 23 in *PIK3CA*, 1 in *AKT3*, and 1 in *mTOR*. Of the 9 negative cases, 5 had residual DNA stock to be analyzed for the vascular pathway genes and a pathogenic variant was identified in 3 of these (2 *GNAQ*, 1 *GNA11*). The remaining 2 negative cases were tested for the RAS-MAPK pathway genes and for 11p15.5 methylation abnormalities, and none were found positive. Finally, of the 39 patients with a clinical suspicion of PROS, 29 were confirmed with a *PIK3CA* pathogenic variant (74.3%), 5 (12.8%) were reclassified to VO with an alternate vascular anomalies gene identified, and 5 (12.8%) were given a diagnosis of presumed PROS based on their clinical phenotypes, because all their molecular tests were negative.

VO

All 12 patients with VO underwent NGS analysis of DNA extracted from the vascular malformation overgrowth area for genes associated with this phenotype, and 6 (50%) tested positive. The remaining 6 underwent NGS analysis of the PI3K/AKT/mTOR pathway genes: 4 of them (66.7%) were found to have pathogenic variants in *PIK3CA* (n = 2) and *PTEN* (n = 2, a brother and sister who inherited the variant from their father with a blunted phenotype). The 2 patients who tested negative underwent the NGS panel for RASopathies genes, with one of them found to have a somatic pathogenic variant in *PTPN11*. Overall, 6 of 12 cases initially suspected with VO were confirmed to have a variant in *GNAS*, *GNA11*, *TEK*, or *RASA1*, whereas 5 (41.7%) were reclassified, including 2 cases of PROS, 2 cases of PTEN-hamartoma tumor syndrome, and 1 case of mosaic RASopathy. One case (8.3%) tested negative for molecular abnormalities.

ILO

All 56 patients with ILO underwent investigation of region 11p15.5 on blood DNA, and of these, 8 (14.3%) were positive: 6 had UPD(11)pat and 1 IC2-LoM and were, therefore, reclassified as affected by a mild form of BWSp, and 1 patient had an IC1-LoM and was diagnosed with a mild form of Silver-Russel syndrome. In 18 cases, patients were either >8 years of age (n = 11) and showed a mild phenotype with no progression of the disease (n = 7), and, therefore, a diagnostic biopsy was considered to be not useful. In the remaining 30 (of 46) negative cases, a diagnostic biopsy was suggested. Six of those patients declined to undergo the procedure; ultimately, 24 underwent MS-MLPA on somatic DNA. Of these, 3 (12.5%) were positive: 2 had an UPD(11)pat and 1 a IC1-GoM. The 21 negative cases underwent NGS analysis of genes involved in the PI3K/AKT/mTOR pathway, revealing 2 positive cases with a variant in *PIK3CA* (11.1%). The remaining 19 negative cases were tested for genes involved in vascular proliferation, and 7 cases had enough residual DNA to undergo the NGS analysis of RASopathies associated genes; all tested negative.

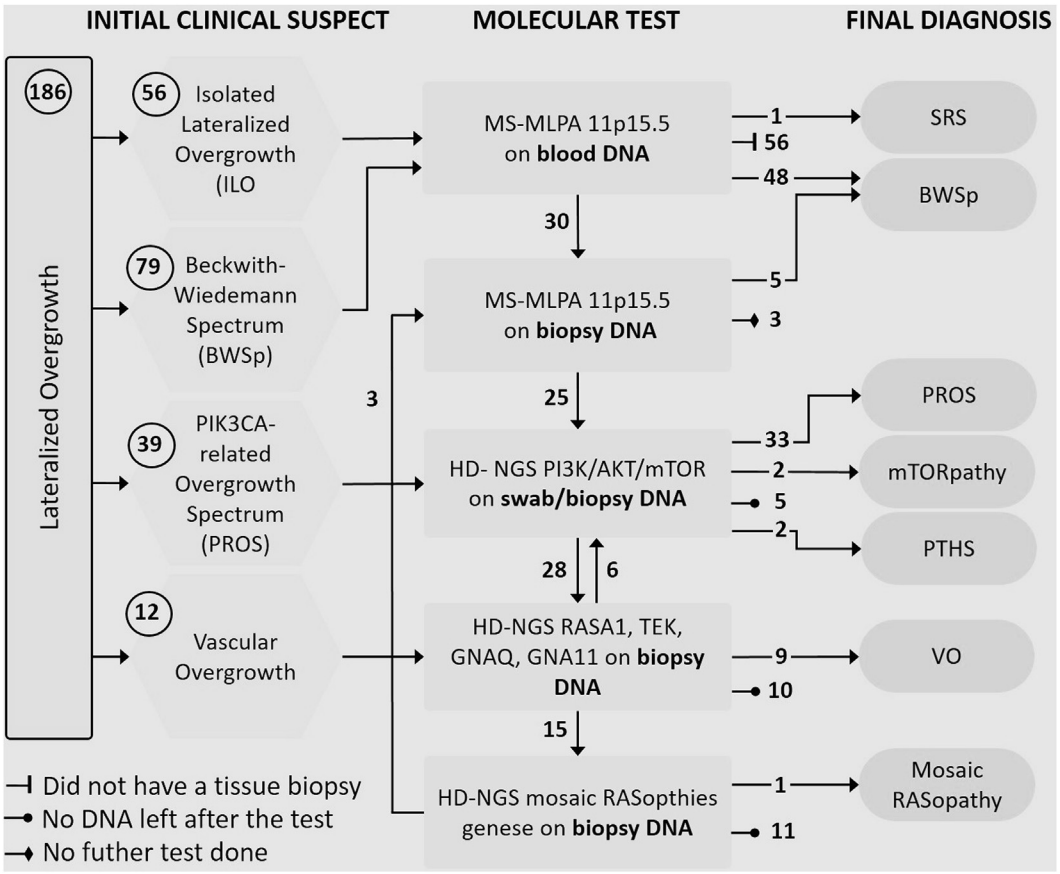

Figure 2. Flow of the molecular tests performed based on the initial suspicion.

Of the 56 patients initially diagnosed with ILO, 12 (21.4%) was reclassified by molecular testing on skin biopsy: 9 cases (16.0%) as BWSp, 1 as Silver Russell syndrome (SRS) (1.9%), and 2 (3.6%) as PROS.

Discussion

Scientific literature on LO is limited, and there is a lack of a systematic diagnostic approach in clinical practice. This study aims to fill this gap by providing a pragmatic approach. Data are provided about LO etiological classifications and, upon retrospective analysis of the molecular workup, a diagnostic molecular workflow is suggested based on the initial clinical suspicion. This work is aimed at selecting the first-tier molecular test and the type of tissue to be analyzed. In cases where the first-tier test is negative, a sequential cascade molecular approach is proposed to gain diagnostic yield. LO cases were divided into isolated and syndromic (BWSp, PROS, and VO) based on physical examination. For patients with ILO or BWSp, first blood and then skin-extracted DNA were tested for 11p15.5 epigenetic anomalies. For patients with PROS or VO, the initial step involved a NGS panel for genes of the PI3K/AKT/mTOR pathway or associated with vascular proliferation, respectively. If the first-tier test was negative, further analysis was conducted using the respective complementary molecular tests due to the well-known phenotypic overlap.

The initial clinical classification was ultimately confirmed at a molecular level in 78 of 130 patients (60.7%) through first-tier tests. The use of a sequential molecular proved rewarding; it resulted in the reclassification of >10% of cases, with significant clinical implications for treatment and management.

This approach to LO as a clinical sign has provided insight into the molecular etiology in both syndromic LO and ILO. LO occurs within the BWSp in 47.8% of cases (28.5% positive, 19.4% negative by molecular tests), within the PROS spectrum in 20.4% of cases (17.7% positive, 2.7% negative by molecular tests), in 7.7% of cases with other VO, in 0.5% of cases in mosaic RASopathies, and in 0.5% of cases in SRS. In 23.1% of cases, ILO is not classifiable despite this comprehensive molecular approach.

It is acknowledged widely that LO is a major feature of BWSp.[5] This study only examined BWSp phenotypes that included LO, meaning those with at least one of the BWSp criteria, including both typical and atypical presentations.[36] The study detected 11p15.5 methylation abnormality in 54.4% of cases. This diagnostic rate is lower than the typical

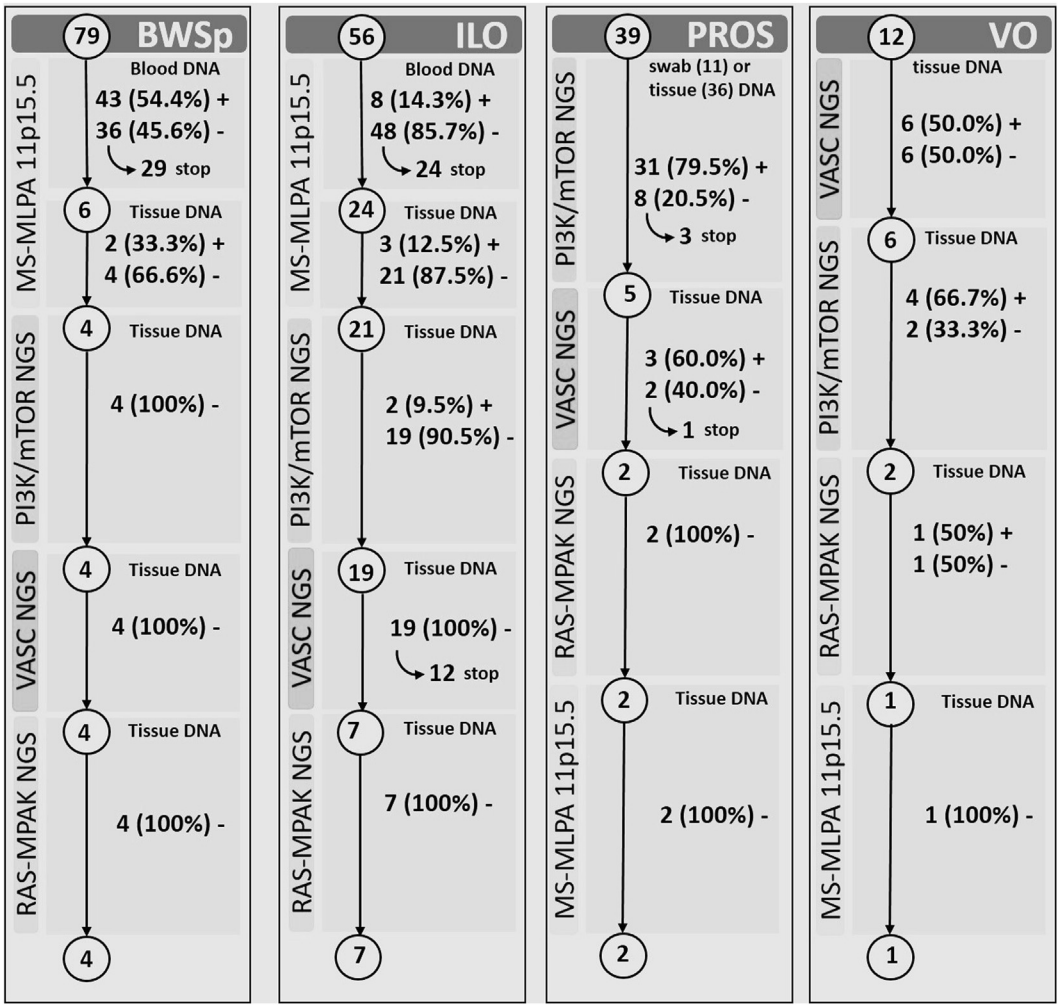

Figure 3. Serial molecular tests on different DNA sources in the 4 groups of patients with LO and respective molecular diagnoses reached in every test.

figure of the BWSp (nearly 85%), likely because this cohort was enriched in milder forms of the BWSp (ie, those with less represented mosaicism), which are typically those with a lower probability of positive molecular tests compared with typical BWSp.[5] Many cases with a negative 11p15.5 analysis on blood were not investigated further because testing tissue-extracted DNA had limited practical implications. Some patients were no longer within the age range for cancer screening, and many others presented clinical features that would allow a clinical diagnosis of BWSp (>4 points on the BWSp score). In cases where methylation studies were conducted on skin biopsy DNA, molecular confirmation was possible in one-third of patients. Therefore, we suggest that repeating the 11p15.5 methylation test on skin biopsy DNA as soon as possible may be beneficial in the setting of a negative blood DNA methylation test and in an appropriately aged patient (who can still benefit from modifications in the screening protocol). Indeed, BWSp has an heterogeneous cancer risk: 2% in IC2-LoM cases, 15% in UPD(pat)11 cases, and 22% in IC1-GoM cases.[20]

The group of patients presenting with PROS had the highest likelihood of a correct initial clinical classification (a pathogenic *PIK3CA* variant was found in 29 of the 39 patients). However, it is important to note the significant phenotypic overlap with VO, as well as with other rarer conditions caused by variants in other genes (*mTOR*, *AKT3*, *PTEN*, and *PTPN11*). Several studies demonstrated that PROS overlaps with these disorders, reflecting the strongly interconnected molecular basis of these diseases at the cellular level.[12,13,15,17,33,37-40] From a practical perspective, it would be more appropriate to use a single comprehensive NGS panel including all the genes in these pathways to optimize time, costs, and skin-extracted DNA consumption which provides limited DNA amounts.

None of the cases with the BWSp phenotype were diagnosed with another disorder at the end of the process, although the number of cases investigated with biopsy

DNA is too small to draw definitive conclusions. Conversely, none of the cases with the PROS or VO phenotype were diagnosed with an 11p15.5 chromosomal region methylation anomaly at the molecular level. The clinical signs of BWSp are distinct from other syndromic LO and allow for accurate differentiation.

In patients with ILO, the positivity rates for 11p15.5 methylation anomalies were similar between MS-MLPA on blood- and tissue-extracted DNA, at 14.5% and 12.3%, respectively. The diagnostic yield of blood DNA testing for 11p15.5 anomalies varies from 5% to 40%.[41-44] Limited data on the yield of tissue-extracted DNA indicate a significant increase in the yield with respect to blood: this depends on both the type of tissue analyzed and the sensitivity of the techniques used.[44-46] Because concordant tissue-DNA analysis is expected in almost all cases with positive blood DNA, it may be debated whether to approach patients with ILO directly through biopsy sample analysis as a first-tier approach to decrease the overall time to reach a molecular diagnosis. This study began with blood DNA testing and, therefore, cannot provide indications regarding a direct approach on biopsy as a first-tier test. Further studies will evaluate this point.

The notable rate of negative molecular tests in cases with ILO remains a significant concern in clinical practice; almost one-quarter still lack a precise diagnosis at the end of the process. This result underscores the need for further investigation into ILO. It is plausible that the current approach fails to detect molecular anomalies owing to inadequate tissue sampling, and that molecular analysis on tissue other than skin may be more appropriate. Future studies could investigate whether there is a difference in diagnostic yield by using deeper tissue samples obtained during surgeries. Additionally, enhancing the diagnostic yield of molecular tests may be achieved by increasing the use of bilateral tissue biopsies. Regarding this, the identification of a case of SRS in this cohort and prior studies raises important considerations.[45] Clinically, it is possible to detect asymmetry between 2 body segments, but it is not always easy to determine which of the 2 asymmetric segments is the abnormal one. Certain syndromes result from lateralized undergrowth of a segment, such as anomalies in 11p15.5 with the corresponding low-expression SRS phenotype and some rare PROS phenotypes (the so-called undergrowth phenotype).[47-50] This observation suggests extending molecular analysis to both asymmetric body areas may be needed to address this possibility fully. This approach, previously described, demonstrated a 30% diagnostic yield and led to change in clinical diagnosis in 40% of cases.[45] Finally, it is important to note that LO may be caused by genetic or epigenetic defects that were not analyzed in this study or are currently unknown. Nevertheless, for the practical management of negative LO cases, a prudential screening program similar to the one adopted for BWSp has been suggested for all of them.[5]

The retrospective nature of this study is one of its intrinsic limitations. This strategy was necessary owing to the rarity of LO, resulting in the inclusion of patients over a broad period of time, some dating back decades. Patients were evaluated at different times and with varying techniques and amount of knowledge. To limit this weakness, all cases were reassessed in the 3 years before publication and updated from a molecular viewpoint as required. Consequently, many suspected cases of BWSp and patients with ILO who tested negative on blood tests did not undergo biopsy DNA testing owing to the patients' age being beyond the screening age, limiting potential benefits. In nonstudy settings, it is important to emphasize the potential benefits of an accurate and early diagnosis to parents of younger children who may wish to postpone the diagnostic skin biopsy as much as possible. Results for analyses conducted on tissues other than skin biopsy (eg, deep tissues obtained during surgery, cells from oral swabs) were not provided. Further studies are required to provide data on the diagnostic yield of various types of analyses on these tissues, which could potentially be superior to investigations conducted on cutaneous fibroblasts.

In summary, this study offers an overview of isolated and syndromic LO from an etiological perspective. It suggests that an approach based on molecular diagnostic tests selected according to the initial clinical presentation is the most rewarding owing to the extreme heterogeneity of this group of disorders. For BWSp, confirmation of the clinical diagnosis can be achieved through MS-MLPA testing for 11p15.5 methylation anomalies on blood and, if negative, on DNA from a skin biopsy or cultured fibroblasts from the LO area. For PROS/VO, it is recommended to initiate the process with skin biopsy DNA and a comprehensive HD-NGS panel. Cases with ILO are complex to approach; they can be caused by either 11p15.5 methylation anomalies or *PI3KCA* variants, 2 conditions with vastly different clinical implications. However, despite a comprehensive molecular dissection, more than one-third of ILO cases remain undiagnosed, clearly indicating a need for further research in this area. ■

CRediT authorship contribution statement

Simone Bellucca: Writing – original draft. **Diana Carli:** Writing – review & editing, Conceptualization. **Andrea Gazzin:** Writing – review & editing. **Stefania Massuras:** Investigation. **Simona Cardaropoli:** Investigation. **Maria Luca:** Investigation. **Paola Coppo:** Investigation. **Mirko Caprioglio:** Writing – original draft. **Roberta La Selva:** Investigation. **Marilidia Piglionica:** Investigation. **Piera Bontempo:** Investigation. **Gemma D'Elia:** Investigation. **Rosanna Bagnulo:** Investigation. **Giovanni Battista Ferrero:** Validation, Supervision, Conceptualization. **Nicoletta Resta:** Investigation. **Alessandro Mussa:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Data curation, Conceptualization.

Declaration of Competing Interest

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