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### ► To cite this version:

Anne-Sophie Jourdain, Florence Petit, Marie-Françoise Odou, Malika Balduyck, Perrine Brunelle, et al.. Multiplex targeted high-throughput sequencing in a series of 352 patients with congenital limb malformations. *Human Mutation*, 2020, 41 (1), pp.222-239. 10.1002/humu.23912 . hal-02393697

**HAL Id: hal-02393697**

**<https://hal.science/hal-02393697>**

Submitted on 15 Sep 2021

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## Multiplex targeted high-throughput sequencing in a series of 352 patients with congenital limb malformations

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/humu.23912.

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### **Abstract**

Congenital limb malformations (CLM) comprise many conditions affecting limbs and more than 150 associated genes have been reported. Due to this large heterogeneity, a high proportion of patients remains without a molecular diagnosis. In the last two decades, advances in high throughput sequencing have allowed new methodological strategies in clinical practice. Herein, we report the screening of 52 genes/regulatory sequences by multiplex high-throughput targeted sequencing, in a series of 352 patients affected with various CLM, over a 3-year period of time. Patients underwent a clinical triage by expert geneticists in CLM. A definitive diagnosis was achieved in 35.2% of patients, the yield varying considerably, depending on the phenotype. We identified 112 single nucleotide variants and 26 copy-number variations, of which 52 are novel pathogenic or likely pathogenic variants. In 6% of patients, variants of uncertain significance have been found in good candidate genes. We showed that multiplex targeted high-throughput sequencing works as an efficient and cost-effective tool in clinical practice for molecular diagnosis of congenital limb malformations. Careful clinical evaluation of patients may maximize the yield of CLM panel testing.

**Keywords:** limb malformation, genetics, molecular diagnosis, targeted high-throughput sequencing

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## INTRODUCTION

Congenital limb malformations (CLM) are common defects, affecting approximately 1:500 new-born. They can be isolated or syndromic and more than 1400 syndromes comprising a CLM are described in the literature. Some of them have a genetic basis and, currently, more than 150 genes or regulatory regions are known to be implicated in limb malformations (Manouvrier-Hanu, Holder-Espinasse, & Lyonnet, 1999; Manouvrier-Hanu, 2012; Zuniga, Zeller, & Probst, 2012). In most cases, the responsible genetic anomaly is a single nucleotide variation (SNV) (Manouvrier-Hanu, 2012). However, copy number variations (CNVs) involving genes or non-coding regulatory elements are also a major cause of CLM (Flottmann et al., 2018).

CLM encompass a wide range of conditions displaying a very large clinical and genetic heterogeneity. Taken individually, each group of pathologies is rare and, as with most rare syndromes, clinical and molecular diagnosis of CLM is not easily assessed. Overlapping features, intra- and inter-familial phenotypic variability, non-specific features and presence of uncommon, unusual or less known phenotypes often characterize CLM, making the diagnosis challenging for the clinical geneticists. Clinical and molecular diagnostic confirmation is nevertheless very important to allow the establishment of a prognosis, to help in the patient support and in genetic counseling. The former gene-by-gene approach carried out by diagnostic laboratories did not help either, since this strategy was very time-consuming and expensive. Nowadays, high-throughput sequencing enabling the simultaneous screening of multiple genes is therefore a very attractive option for the clinical practice (Abou Tayoun, Krock, & Spinner, 2016). Two approaches are available: whole exome/genome sequencing (WES/WGS) or targeted high-throughput sequencing of gene panels. For diagnosis purposes, WES/WGS have still significant limitations due to the costs and time and specialized computing infrastructures that are needed for analysis and data storage. Targeted sequencing is a time and cost-saving strategy to screen from dozen to hundreds of genes.

To our knowledge, no study using high-throughput sequencing in CLM patients has been published so far. In 2009, Furniss *et al.* reported the genetic screening of 13 limb genes in 202 CLM patients, causative alterations were identified in 11% of cases (Furniss et al.,

2009). Prior to 2014, our laboratory carried out the analysis by Sanger sequencing of only 20 genes (*BHLHA9*, *BTRC*, *GDF5*, *HOXA13*, *HOXD13*, *IHH*, *LMX1B*, *NOG*, *PITX1*, *PTHLH*, *ROR2*, *SALL1*, *SALL4*, *SF3B4*, *TBX3*, *TBX4*, *TBX5*, *TP63*, *WNT5A*, *WNT10B*). An additional regulatory sequence (*ZRS*) was also analyzed by Sanger sequencing and copy number determination. The diagnosis yield was 27.5% (data not shown). Since 2016, we had performed next generation sequencing (NGS) by target capture, enabling the simultaneous detection of SNPs and CNVs. We routinely analyzed a panel of 50 genes and 2 regulatory sequences, representing a good compromise for cost and ease of data analysis. We included human genes associated with known Mendelian diseases comprising a CLM (Supp. Table S1: list of genes, associated CLM and MIM#). All demands for genetic testing were carefully assessed clinically. For all patients fitting CLM clinical criteria, genetic testing by NGS was performed. However, in the case of isolated Split Hand/Foot Malformation (SHFM) with or without Long bone Deficiency (SHFLD), first tier analysis was the copy number determination of *BTRC* and *BHLHA9* by quantitative PCR (qPCR). Indeed, copy-number gains of either locus are the major causes for SHFM/SHFLD (Carter et al., 2017). If the qPCR was negative, NGS-based panel testing was performed as a second tier analysis, except for patients affected with only one split hand. In the latter, the analyses were discontinued since a genetic cause is unlikely.

In this article, we report on the retrospective analysis of a series of 352 patients presenting CLM and referred to our routine laboratory for diagnosis over a period of 3 years between March 2016 and February 2019. A causal variation was detected in 35.2% of these patients, with a great variability of the diagnosis yield among the different types of limb malformations. We show that the implementation of a gene panel has increased our diagnostic efficiency. In some cases, this allowed us to extend the clinical spectrum or redirect the diagnosis.

## **MATERIALS AND METHODS**

### **Editorial Policies and Ethical Considerations**

Analyses were performed on a diagnosis basis following the bioethics rules of French law.

### **Patients**

In this study, we included a total of 352 unrelated probands with genetically undiagnosed CLM that have been analyzed in our laboratory between 03/01/2016 and

02/28/2019. All patients were referred by clinical geneticists. Clinical and family history details were collected as well as informed consent for genetic studies.

All patients have clinical features suggestive of a disorder associated with one of the 52 targets (genes/regulatory sequences) analyzed, although the features may not be completely typical or fulfill the clinical criteria of a specific syndrome at the time of recruitment.

The clinical data for each case was reviewed by a clinical geneticist expert in limb malformations, in order to classify the CLM in 7 groups (Figure 1), further subdivided in several categories: radial anomalies (Holt-Oram Syndrome, Okihiro Syndrome, Nager Syndrome, *RECQL4*-disorders, Thrombocytopenia-Absent Radius Syndrome, Townes-Brocks Syndrome, unclassified), ectrodactyly (Split hand or Split-Foot, SHFM, SHFLD, Ectrodactyly-Ectodermal dysplasia-Clefting (EEC) syndrome), brachydactylies (Type A, Type B, Type C, Type E, Robinow syndrome, Tricho-Rhino-Phalangeal syndrome, unclassified), polydactylies (preaxial, postaxial, synpolydactyly, unclassified), reduction anomalies (transversal, longitudinal, phocomelia/amelia, Adams-Oliver Syndrome, unclassified), fusion anomalies (syndactylies, multiple synostosis), patella hypoplasia (Nail-Patella Syndrome, Small-Patella Syndrome). Note that the reduction anomalies group does not comprise the radial reduction defects, which are classified in the radial anomalies, nor the reduction of the central rays of the autopod, which are classified in the ectrodactyly group.

### **DNA extraction**

Genomic DNA was extracted from blood samples using the Chemagic Star (Hamilton, Chemagen) with B1k kit (PerkinElmer) according to the manufacturer's instructions. When only fetal tissue was available, DNA was extracted using the Chemagic Prepito-D with Prepito Cyto Pure kit (PerkinElmer).

### **Quantitative PCR**

For patients with isolated SHFM or SHFLD, quantitative real time PCR (qPCR) assays in the *BHLHA9* and/or *BTRC* genes were performed. Likewise, each CNV identified by NGS (BMP2 enhancer, *NOG*, *SF3B4*, *RBM8A* and *TP63*) was validated by qPCR. The reaction was performed with the PowerSyber Green technology

according to the manufacturer's protocol (ThermoFischer Scientific) on QantiStudio 7 (Thermofischer Scientific). Quantification of the target sequences was normalized on 2 control genes. The relative copy number was determined on the basis of the comparative  $\Delta\Delta C_t$  method using a normal control DNA as the calibrator.

### **Targeted Sequencing workflow and analysis**

#### **Library preparation and sequencing**

Genomic DNA have been quantified using the Quant-iT PicoGreen® dsDNA assay kit (Thermofischer scientific) on the Xenius SAFAS Monaco and normalized in nuclease free water at 25ng/ $\mu$ L in a final volume of 5 $\mu$ L. Libraries were prepared as described in the manufacturer's protocol SureSelect QXT (Agilent). Paired-end sequencing (2x150bp) was performed on the MiSeq sequencer (Illumina), on a standard FlowCell.

#### **Targets**

Clinical panel was developed through a collaborative process involving both the molecular diagnosis laboratory and the clinical experts in CLM at our institution. Genes were selected for their implication in limb malformations based upon a review of the medical literature and public databases like OMIM ([www.omim.org](http://www.omim.org)) and PubMed ([www.ncbi.nlm.nih.gov/pubmed](http://www.ncbi.nlm.nih.gov/pubmed)). With SureDesign (Agilent<sup>TM</sup>) software, we targeted all exons of the reference transcripts from the Human Gene Mutation Database (HGMD) to design the SureSelect QXT probes. The content of the panel was continuously updated and, over time, 3 versions of the panel have been developed (V1, V2, V3), detailed in Supp. Table S1.

#### **Bioinformatics analysis**

The FastQ data were aligned on the human genome reference (GRCh37-hg19) using the Burrows-Wheeler Aligner algorithm (BWA, v0.7.15-r1140) and variant calling was performed using Genome Analysis Tool Kit (GATK, v3.7). The variant call format (VCF) was annotated using VarScan (v2.3.6) and integrated in a homemade database called DVD (Bioinformatics, CHU-Lille). The open source softwares CNVkit v0.8.5 (Talevich, Shain, Botton, & Bastian, 2016) and CANOES (Backenroth

et al., 2014) were used to infer and visualize copy number from targeted DNA sequencing data. Bioinformatic filters were applied to prioritize the variants' type, frequency in public and in in-house polymorphic databases. Variants outside exons +/- 25 bp intron sequence were excluded. Only variants resulting in non-synonymous amino acid changes (missense, nonsense, exonic insertion/deletion) and intronic splice variants were analyzed. Frequency data was based on 1000 Genomes (<http://www.1000genome.org>), Exac Browser (<http://exac.broadinstitute.org>), GnomAD (<https://gnomad.broadinstitute.org/>) or dbSNP (Build 137, NCBI). Pathogenicity predictions for missense variants were made using the prediction programs Polymorphism Phenotyping v2 (Polyphen-2), Sorting Tolerant from Intolerant (SIFT) and Mutation Taster through Alamut Visual v.2.11.0 (Interactive Biosoftware, Rouen, France) (Supp. Table S1). The classification also included reports in the Human Gene Mutation Database (HGMD, <http://www.hgmd.cf.ac.uk/ac/index.php>), OMIM (Online Mendelian Inheritance in Man) data, ClinVar (<https://www.ncbi.nlm.nih.gov/clinvar/>) and the relevant literature. Effects on splicing were predicted using the splicing module of Alamut v.2.11.0, questioning the following programs: SpliceSiteFinder-like, MaxEntScan and NNSPLICE (Supp. Table S2).

### **Description and interpretation of variants**

Variants were described according to the recommendations of the Human Genome Variation Society ([www.hgvs.org/](http://www.hgvs.org/)) and submitted to the ClinVar database (SUB5823379). Their interpretation followed the classification system recommended by the American College of Medical Genetics and Genomics (ACMG) (Richards et al., 2015).

### **Confirmation using other methods**

#### **Sanger sequencing**

Sanger sequencing was used to reanalyze targeted exons with low or no coverage, to confirm the nucleotide variations identified by NGS and for segregation analyzes when familial samples were available. Amplicon fragments were sequenced using the ABI Prism 3730XL Genetic Analyzer (Applied Biosystems, Courtaboeuf, France).



## Multiplex Ligation Probe Amplification

Multiplex Ligation-dependent Probe Amplification assay (MLPA) was performed to confirm deletion/duplication in *SALL4*. We used the commercially available MRC Holland Salsa kit P180 according to the manufacturer's protocol. Results were analyzed using the Coffalyser v.0.1407221 software (MRC-Holland)

## RESULTS

### Limb malformation panel performance

The SureSelect capture of genes involved in limb malformations (Supp. Table S3) yielded a high coverage in the targeted regions with a mean depth of 937.9X. On average, 98.9% of the targeted regions were covered with a read depth of a least 30X. Only 1.1% of the targeted regions had coverage below 30X or no coverage. Those are exclusively first exons due to high GC content (*DOCK6*, *FGFR3*, *HOXA13*, *NOTCH1*, *PITX1*). One hundred percent of the bases in targeted regions showed an excellent quality value (QC>30).

### Molecular findings in the limb malformation cohort

Three hundred and fifty-two probands with limb malformations were analyzed between March 2016 and February 2019. Of these, 314 patients had a NGS analysis for the CLM panel while 38 patients with isolated SHFM/SHFLD had *BHLHA9* or *BTRC* qPCR as a first-line test. For twelve of the latter, a NGS analysis was performed as a second-line test, raising the NGS-screened patients to 326. The results are summarized in Figure 2.

Variants were identified in 31 of the 52 genes/regulatory sequences analyzed. The most frequent anomalies in the cohort, in descending order, were variants in *LMX1B*, *GLI3*, *TBX5*, *TP63*, *RBM8A*, *GDF5*, *TRPS1*, *SALL1*, *IHH*, *DOCK6*, *BHLHA9*, *SALL4*, *SF3B4* and 10q24 duplication involving *BTRC*. Overall, 160 candidate variants were found in 152 index patients (43.2%), distributed as follows: 134 SNVs (83.8% of all variants) and 26 CNVs (16.2%). Among them, 72 were novel variants.

After applying the ACMG criteria, 130 variants (104 SNV and 26 CNV) (81.3%) were classified as pathogenic or probably pathogenic and 30 SNV (18.7%) were considered of uncertain significance (VUS). Among VUS, eight were reclassified into probably pathogenic variants after familial segregation results and, finally, 124 patients received a confirmed diagnosis. Table 1 summarizes the pathogenic and likely pathogenic variants identified in the cohort. Identified variants could explain the observed clinical phenotypes in all cases, giving an overall diagnostic yield of 35.2%. Diagnosis yield was higher in syndromic or familial CLM cases (Figure 3). In 7 probands affected with autosomal recessive CLM, only one molecular pathogenic hit has been identified.

For 22 VUS (22 patients), further investigations are required (familial segregation, functional assays...) to assess their pathogenicity. However, these VUS are likely candidates since 9 (40.9%) are located in the gene initially suspected and 22 (100%) are consistent with the clinical presentation. Most of them (15/22) are missense variants which had not been previously reported in medical literature and databases. Among these variants, 100% (15/15) had a CADD (Combined Annotation Dependent Deletion) score between 20 and 30 (Kircher et al., 2014). Seven variants are located in candidate non-coding regions: 3 of them in the ZRS (regulatory region of the *SHH* gene) and 4 of them in the non-coding region of *RBM8A* gene.

To have a better view of the diagnosis yield for each type of malformation, patients were classified into 7 groups according to the clinical entryway, after reviewing by a geneticist specialized in limb malformations. The results are summarized in Table 2. Diagnostic yield was found to be extremely variable between the different phenotypic groups. Among this cohort, it was highest in the patella hypoplasia group (95.2%) and even rose to 100% in the subgroup of patients with Nail Patella syndrome. In contrast, the diagnostic yield for reduction anomalies (42 patients screened) was very low (9.5%).

In 228 cases (64.8%), no definite molecular diagnosis could be confirmed, either because no molecular variation was identified, or a VUS was identified, or only one pathogenic variant could be identified in recessive condition. No evidence for oligogenic inheritance has been found in this cohort.

## Radial anomalies

Overall diagnostic yield was 32.7% for the 101 patients of the radial anomalies group. This group comprises several different syndromic conditions (Holt-Oram Syndrome, Okihiro Syndrome, Townes-Brocks Syndrome, TAR Syndrome, Nager Syndrome and *RECQL4* disorders). However, the diagnostic yield is barely similar (30 to 50%), being highest for TAR and Nager Syndromes (Table 2).

The candidate variants identified were usually found in genes suspected prior the analysis. In 3 cases however, the NGS results allowed to reconsider the initial clinical hypothesis.

For two patients with duplicated thumb together with multiple congenital anomalies, we found variants in the *PUF60* gene. This gene encodes a poly-U-binding splicing factor involved in pre-RNA splicing and transcription, through the interaction with other factors such as SAP49 encoded by *SF3B4*. It was recently described in patients with multiple congenital anomalies, growth retardation and intellectual disability. Limb malformations were reported in some cases but not precisely described in the literature. In case NGS226, we initially suspected an Okihiro Syndrome because of an associated congenital heart defect, bilateral coloboma and severe scoliosis. A novel *de novo* frameshift variant was identified in one of the RNA recognition motif domains of the protein, where all reported variants cluster. In case NGS309, a Townes-Brocks Syndrome was hypothesized because the duplicated thumb was associated with a Sprengel anomaly with fusion of cervical vertebrae, anal anteversion and a branchial sinus (Figure 4). A novel *de novo* splicing variant was identified in intron 5 of the *PUF60* gene, responsible for the out-of-frame insertion of 11bp confirmed by the transcript analysis on lymphoblastoid cell line from the patient.

An Okihiro Syndrome was initially suspected in case NGS228, affected with bilateral hypoplastic thumbs, 2-3 syndactyly of toes, unilateral kidney agenesis, and small dysplastic ears (Figure 4). However, a probable pathogenic missense variant was found in *FGFR2* gene, involved in the Lacrymo-Auriculo-Dento-Digital Syndrome and already reported in the literature (Table 2). The variant appeared to be inherited from the mother, who had a flexion defect of both thumbs. No lacrymal duct stenosis or deafness was reported in the proband or his mother.

We studied 11 patients typical for TAR Syndrome, an autosomal recessive condition due to biallelic variants in *RBM8A* gene. The diagnosis was confirmed molecularly in 4 of them. In 6 patients, a *RBM8A* deletion was identified, without any second hit in 2 cases, or with a VUS on the other allele in 4 cases. These candidate VUS are located in the non-coding regions of *RBM8A* and functional studies are on-going. Therefore, our diagnosis yield of 36.4% may increase as far as 73% after functional validation.

Finally, 5 patients with radial anomalies could not be classified in a known syndromic condition. No candidate variant was identified in these unclassified patients.

### **Ectrodactyly**

In the ectrodactyly group, 50 patients were screened and variants were detected in 21 cases (42%). The most frequent anomalies were *TP63* variants, *BHLHA9* duplications and 10q24 duplications involving *BTRC*.

In this cohort, 38 patients (76%) with an isolated CLM first underwent screening by qPCR for *BHLHA9* and *BTRC* copy-number gain. *BHLHA9* duplication was found in 6 patients (6/38, 15.8%). Phenotype was highly variable, ranging from bilateral split hand to SHFLD. Also, one patient had an isolated monodactyly of one hand. *BTRC* duplication was found in 4 cases (4/38, 10.5%) with SHFM. Among the 28 negative cases: 16 had an isolated unilateral split hand and the analyses were discontinued; 12 had ectrodactyly involving at least 2 extremities, and the analyses were continued with the NGS panel.

The NGS panel was performed overall in 24 patients affected with ectrodactyly (12 had isolated CLM with a negative screening for *BHLHA9* and *BTRC* copy-number gain, 12 had syndromic ectrodactyly).

Among the 12 syndromic cases, 9 had a phenotype consistent with EEC Syndrome, while 3 patients had SHFM together with various congenital anomalies (diaphragmatic hernia in case NGS168, duodenal atresia and congenital heart defect in case NGS147, mandibulo-facial dysostosis and deafness in case NGS232). *TP63* mutations were identified in 8 patients with EEC syndrome (88.9%, 8/9) but also in two patients with isolated SHFM of the 4 extremities. One proband was a 29 year-old female (NGS279). However, the second proband was a termination of pregnancy at

15 WG (NGS274). Fetal autopsy did not show cleft lip or palate, but ectodermal dysplasia could not be studied. In one case affected with bilateral split foot (NGS276), a nonsense variant in *WNT10B*, inherited from the asymptomatic mother was identified. While clearly deleterious, we chose to consider that the molecular diagnosis is not established in this proband, since the second hit has not been identified. However, we reported recently that heterozygous *WNT10B* carriers may be symptomatic (Brunelle et al., 2019). Therefore it is highly likely that this variant plays a role in the limb phenotype in this case.

No variant in *FGFR1*, *DLX5* or *DLX6* was found in this series of 24 patients who underwent the NGS screening.

### **Brachydactyly**

Overall diagnostic yield was 39.7% for the 63 patients of the brachydactyly group, but highly variable between the subgroups (Table 2). The yield is particularly low in type E brachydactyly (BDE), with only 2 positive diagnoses in 19 patients (10.5%). Interestingly, one patient initially referred for non-syndromic familial BDE (NGS079, Figure 4) carried a likely pathogenic variant in *TRPS1* segregating in several affected relatives. Fine clinical evaluation of the affected father, grand-mother and aunt, together with progressive dysmorphic features and hair anomalies in proband's follow-up, allowed to review the diagnosis to Tricho-Rhino-phalangeal Syndrome.

*TRPS1* appeared to be the most frequently causative gene in the brachydactyly, together with *GDF5* gene (responsible for Type-C Brachydactyly, BDC). A duplication of the *BMP2* enhancer was found in two probands. One of them was affected with Type A2 Brachydactyly (BDA2) (NGS027), consistent with the associated phenotype reported in the literature, while the other proband was affected with BDC (NGS050). Surprisingly, no variant was found in *PTHLH* gene.

In 8 patients, the brachydactyly phenotype could not be classified. The molecular analysis identified the causative gene in only one of them (NGS081). This proband carried a *de novo* nonsense variant in *GLI3* and had short middle and distal phalanges associated with bilateral triphalangeal thumbs. The feet had large halluces and clinodactyly of toes with a median plantar crease (Figure 4). There was no macrocephaly and the corpus callosum was present. This phenotype was intriguing,

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since triphalangeal thumbs had never been reported in other *GLI3*-mutated patients to our knowledge. After the molecular results, additional investigations showed a bifid epiglottis and a hypothalamic hamartoma, compatible with Pallister-Hall syndrome.

Finally, VUS candidates were identified in 6 patients in *IHH*, *GDF5* and *HDAC4* genes. Familial segregation studies may help the interpretation.

### **Polydactyly**

Fifty-one patients affected with different types of polydactyly were screened. The major causative gene was *GLI3*, with variants found in preaxial, postaxial or mixed polydactylies. The diagnostic yield reaches 70% in the latter subgroup. We identified 12 pathogenic or likely pathogenic variants in *GLI3* and one candidate VUS. In the preaxial subgroup, we also found 3 variants in the ZRS (limb-specific *SHH* enhancer). These were novel variants affecting highly conserved nucleotides during evolution and unreported in the databases. Due to their location in a non-coding region, their functional consequences will be assessed by enhancer assays. So far, they have been classified in the VUS category. Interestingly, a VUS in *GLI2* was found in a female patient presenting with postaxial polydactyly affecting all four extremities (NGS055). Additionally, the proband had an occult cranium bifidum with a head circumference at +2SD. The VUS segregated in the father and another child who also had postaxial polydactyly of the four extremities. Together, these findings are consistent with the deregulation of the *SHH* signaling pathway as the main molecular cause for polydactyly.

Furthermore, in the female proband NGS249 and her mother, there was a frameshift variant in *TBX3*. Both patients had a unilateral postaxial polydactyly, associated with clinodactyly and synostosis of the middle and distal phalanges of the 5<sup>th</sup> fingers (Figure 4). *TBX3* is responsible for Ulnar-Mammary Syndrome, an autosomal dominant disorder characterized by posterior limb deficiencies or duplications, apocrine/mammary gland hypoplasia and/or dysfunction, abnormal dentition, delayed puberty in males, and genital anomalies. No extra-limb feature was found in this family.

Synpolydactyly is a particular subgroup where polydactyly is associated with extended syndactylies. The only causal variants found in this sub-group affect the *HOXD13* gene in 50% of patients.

### **Reduction anomalies**

As mentioned earlier, it is important to note that this group excluded the radial reduction defects and the SHFM/SHFLD. Among the 42 patients included, the diagnosis yield was low at 9.5%.

In particular, we did not find any causative or candidate variant in longitudinal/transversal defects or in phocomelia/amelia patients. Chromosomal microarrays (CMA) have been performed in 96% of these patients, revealing no candidate CNV. Some patients were affected with sporadic conditions of unknown etiology like Gollop-Wolfgang Complex or Femoral-Facial Syndrome, which may be due to unknown genes so far or to different mechanisms.

Conversely, we found the causative molecular anomalies in 44.4% (4/9) of cases from the Adams-Oliver subgroup. An additional patient carried a candidate missense VUS in *NOTCH1* for which the familial segregation is in progress. All 9 patients were typical, affected with scalp defects and terminal transverse defects of the hands and/or feet (Figure 4). For one of them, the cutis aplasia had been overlooked at the first examination, and only noticed when reviewing the patient after the molecular diagnosis. Of the 5 genes known so far to cause Adams-Oliver Syndrome, the most frequently involved was the *DOCK6* gene, responsible for an autosomal recessive form.

### **Fusion anomalies**

In this group, 24 patients were screened because of syndactyly (15 patients) or multiple synostosis (9 patients). The fusion anomaly was isolated in 9 cases (37.5%). Variants were identified in 9 cases: 6 pathogenic or probably pathogenic variants and 3 VUS.

*NOG* was the major causative gene for multiple synostosis (3/5 patients, of which one case harboring a *NOG* gene deletion). These patients presented with broad thumbs



and hallux, carpal fusion and synostosis of the 4<sup>th</sup> and 5<sup>th</sup> fingers phalangeal or metacarpo-phalangeal joints (NGS287, NGS291, NGS296), associated with conduction deafness due to stapes ankylosis in one of them (NGS291, *NOG* deletion). We also found a *de novo* likely pathogenic variant in *FGF9* in one case affected with bilateral synostosis of the thumb phalanges, absence of the hallux first phalanges and bilateral dislocation of the radial head (NGS283).

In patients with syndactyly, variants were identified in *FGFR2*, *GLI3* and *DLL4* genes. Case NGS282, carrying a *de novo* likely pathogenic variant in *FGFR2*, had a typical Pfeiffer syndrome with craniosynostosis and complete membranous syndactyly 2-5 of fingers, 2-3 of toes. In case NGS257, we identified the pathogenic nonsense p.(Ser372\*) in *GLI3*. The patient presented with bilateral 2-3-4 syndactyly of fingers, bilateral 1-2-3-4 syndactyly of toes and head circumference was +2SD (Figure 4). The variant was inherited from the mother with similar clinical features. In case NGS216, we identified a missense VUS affecting the zinc finger domain of the *GLI3* protein. The proband had mesoaxial syndactylies of hands and feet, but normal head circumference. This phenotype segregated in the family, following an autosomal dominant inheritance. Segregation studies may help to reclassify the variant. In both families, an unusual phenotype for *GLI3* was observed, with mesoaxial syndactylies and absence of polydactyly.

Interestingly, we found a missense VUS altering a highly-conserved residue of *DLL4*, in a patient affected with bilateral 3-4 syndactyly of hands and terminal transverse defects with anonychia of the 4<sup>th</sup> fingers (NGS086). While these distal reduction defects are consistent with the diagnosis of Adams-Oliver Syndrome, the patient had no scalp defect. Familial segregation study may help to interpret these results.

### **Patella hypoplasia**

The patella hypoplasia group, comprising 21 patients, harbored the highest diagnostic yield of the cohort (95.2%). Note that this group only comprised Nail-Patella and Small Patella Syndromes. The *RECQL4*-disorders, that may also comprise a patella hypoplasia, have been classified in the radial anomalies group, because this is usually the prominent feature.



All 18 cases affected with Nail-Patella Syndrome have been resolved, with the identification of a pathogenic or likely pathogenic variant in *LMX1B*.

Among the 3 Small-Patella Syndrome cases, the same nonsense pathogenic variant p.(Arg261\*) was identified in *TBX4* confirming the diagnosis in two unrelated probands. One of them was a male patient (NGS281) presenting with bilateral hypoplastic patellae responsible for recurrent dislocations and sandal gaps. Interestingly, pelvis X-rays were normal, showing no defect of the ischiopubic junction ossification at 15 years of age. There was no pulmonary hypertension. In the third proband (NGS100), a candidate missense variant was identified in the T-Box domain of *TBX4*, affecting a highly-conserved residue and never reported previously in the databases. This proband had bilateral hypoplastic patellae responsible for dislocations in late childhood, and a defect of the ischiopubic junction ossification at 37 years old. There was no pulmonary hypertension. Familial segregation study may help the interpretation.

## DISCUSSION

CLM are a diagnostic challenge due to their variable expressivity, clinical overlap between the different syndromes (e.g. radial anomalies) and their genetic heterogeneity. NGS approaches are increasingly being used for genetic diagnosis in routine clinical practice and many publications report successful use in heterogeneous disorders such as inborn errors of metabolism (Yubero et al., 2016), neuromuscular disorders (Stehlikova et al., 2017), intellectual disability (Martinez et al., 2017). To our knowledge, no report of the diagnostic impact in patients affected with CLM using high-throughput sequencing has been published so far. In this study, we report on our data obtained on a series of 352 patients with CLM, using a strategy including quantitative assays and multigenic high-throughput sequencing panel.

The strategy adopted by our multidisciplinary team was used to optimize the turnaround time and diagnostic performance and to maximize the potential for a differential diagnosis without the need for multiple testing, together with keeping the cost down. Since no commercial gene panel was available for CLM, we developed a custom panel of 52 gene/regulatory targets, to provide comprehensive coverage of known CLM-associated genomic regions. We chose to target genes in which variants were known to cause CLM, keeping their number low to facilitate the analysis. We

selected the genes responsible for isolated or syndromic CLM through the Online Mendelian Inheritance of Man database. In the syndromic conditions, only genes for which CLM is a prominent feature in the phenotype have been selected.

This NGS-based panel testing approach has significantly simplified our workflow in the diagnostic laboratory. Before 2016, Sanger sequencing was performed for up to 21 genes and regulatory regions. In most patients, multiple genes had to be screened sequentially given the genetic heterogeneity of most CLM. Multiple targets can be screened at once, including frequent, rare or differential genes.

The choice of the capture-based enrichment enables us to analyze sequence variants as well as CNVs. With bioinformatic algorithms, CNV detection with NGS is easy and can replace traditional methods such as quantitative PCR or MLPA (Yao, Yu, Qing, Wang, & Shen, 2019). The importance of CNV detection in CLM has been confirmed by numerous studies (Carter et al., 2017; Flottmann et al., 2018). Our strategy allows the detection of CNVs in all 52 targeted regions, when formerly CNV determination was possible in only a very limited number of genes. In the present series, CNVs were identified in 7.4% (26/352) of patients. Among them, 14 CNVs out of 26 identified (54%) would not have been identified with our previous workflow, due to the absence of home-made and commercial tests available (*RBM8A*, *TP63*, *SF3B4*, *NOG*, *BMP2* enhancer). However, we have made the choice to keep on performing q-PCR for *BHLHA9* and *BTRC* as a first-line test in ectrodactyly because of its rapidity and low cost. However, given the low diagnostic rate in this phenotype group, this should be reevaluated. A NGS-first approach would certainly be interesting for these patients as well.

Overall, in this series of 352 patients, 138 pathogenic or likely pathogenic variants were identified, among which 26 CNVs, and 22 variants of uncertain significance. Our approach revealed to be a useful strategy, providing a molecular diagnosis in 35.2% of patients, similar to other heterogeneous conditions (50% for inborn errors of metabolism (Yubero et al., 2016), 47.3% for myopathies and muscular dystrophies (Stehlikova et al., 2017), 39% for intellectual disability (Martinez et al., 2017), 28.5% for epileptic encephalopathy (Kothur et al., 2018), 28.1% for disorders of sexual development (Fan et al., 2017), 24.5% for developmental eye disorders (Patel et al., 2019), 20% for primary arrhythmia

syndrome and cardiomyopathy (Robyns et al., 2017), 18% for inherited polyneuropathy (Wang et al., 2016)). A 7.7% increase was observed in our diagnostic performance, compared to our previous workflow (diagnostic yield 27.5% before 2014, data not shown). This increased yield may be explained by the additional targeted regions as well as by the possibility for CNV detection. Also, we can predict an improvement in assessing differential diagnoses thanks to the panel screening, compared to the candidate gene approach required for the Sanger sequencing.

VUS were identified in 6.2% of patients, with the need for additional investigations (familial segregation, functional assays, *in vivo* model...). Most of them could potentially be reclassified as likely pathogenic variants if segregation analysis could be performed in the family.

The interpretation of variants needs a tight collaboration between clinical geneticists and molecular biologists, especially when managing CLM patients for whom the molecular cause might be heterogeneous. We observed that the diagnostic yield is highly dependent on the clinical selection of patients. For example, in typical phenotypes or easily recognizable syndromes (e.g. Nager Syndrome, EEC Syndrome, Tricho-Rhino-Phalangeal Syndrome, Robinow Syndrome, Nail Patella Syndrome...), the yield may raise up to 50 or even 100%, consistent with what is described in the literature for the targeted analysis of several conditions (Nager syndrome (Bernier et al., 2012), Nail-Patella syndrome (Ghoumid et al., 2016), SHFM (Sowinska-Seidler, Socha, & Jamsheer, 2014), EEC syndrome (Rinne, Hamel, van Bokhoven, & Brunner, 2006)...). Careful clinical examination prior to and after molecular testing is crucial in some cases to assess the variant pathogenicity. Typical features may not have been explored initially or may have been overlooked (e.g. cutis aplasia in Adams-Oliver Syndrome, hair anomalies in Tricho-Rhino-Phalangeal Syndrome...). Conversely, it was observed that when no clinical hypothesis could be assessed (unclassified sub-groups), the diagnosis yield is close to zero (except for the brachydactyly group, where the phenotypes are more difficult to classify in each sub-group). The relevance of the panel screening should therefore be discussed in those cases.

Despite the clinical utility of the limb malformations panel, most cases (56.8%, 200/352 cases) are negative for a germline variant. The failure to achieve a diagnosis in 228 patients confirms the need for further developments. For each case, a

multidisciplinary discussion was held in an attempt to define further testing. In some cases, phenotyping was limited by the lack of clinical information, X-rays and pictures or the poor quality of the latter.

Limb development is a complex process requiring the precise regulation and inter-connection of multiple signaling pathways, making it highly sensitive to environmental factors (parental exposure to teratogenic agents, *intrapartum* events...). For 7 patients, an environmental or non-genetic cause was suspected (maternal diabetes, contraceptive or valproate exposition during pregnancy, amniotic bands, vascular cause) and the molecular analyses were motivated to rule out a genetic cause, in the context of genetic counseling.

For certain patients, a molecular diagnosis was subsequently made. Two cases were affected with radial anomalies and cardiac septal defects (NGS087 and NGS290). The first suspected diagnosis was Holt-Oram Syndrome but, given the absence of *TBX5* anomaly after the NGS screening, a chromosomal breakage analysis was performed, showing the instability typical for Fanconi anemia disease. In both cases, the diagnosis was able to be confirmed by molecular testing through a high-throughput sequencing panel dedicated for genetic anemia. Given the large genetic heterogeneity (more than 20 genes) and the availability of the chromosomal screening test, we chose not to include the causative genes in our CLM panel. For the same reasons and also because the limb features are not prominent in the clinical picture, the causative genes for Diamond-Blackfan anemia were not included.

Such a high negative rate in individuals with significant congenital anomalies suggests that additional genes may be involved. However, it is unlikely that exome sequencing would significantly increase the diagnostic rate in the negative patients, since *trio* analyses usually have a similar yield in heterogeneous patient populations (literature review: 34.3% (Dragojlovic et al., 2018)). Also, this approach would generate more unsolicited findings and VUS, therefore increasing the cost and time spent on the analysis. Furthermore, the technical quality of the NGS panel was high, demonstrating excellent coverage and read depth for most genes, compared to the results obtained by exome sequencing for many genes of the panel (e.g. low coverage for *BHLHA9*, *EOGT*, *HOXA13*, *TBX5*...). To test this approach, we performed whole exome sequencing in 31 negative patients (17 cases with radial anomalies, 5 with

ectrodactyly, 4 with brachydactyly, 3 with reduction anomalies and 2 with patella hypoplasia). In all cases, no definite molecular diagnosis could be confirmed (data not shown), suggesting the implication of variants located in non-coding regions such as UTRs, in deep intronic or in regulatory regions. Indeed, many CLM are due to variants in non-coding regions that would be missed by exome sequencing (e.g. in the preaxial polydactyly sub-group, 15% of patients carry a variant in the ZRS). Therefore, genome sequencing that offers the opportunity to explore the full contribution of non-coding variants, including SNV, CNV or structural variants, could be a good choice in the near future, like it has been shown in other pediatric diseases (Lionel et al., 2018).

Genomic disorders may have been insufficiently studied. CMA was performed in 89% of the patients before the NGS screening, while balanced chromosomal rearrangements have not been studied in most of them.

The major concern for the targeted panel is the need for frequent updates, which would not be necessary with the exome sequencing approach. During the past 3 years, we set up 3 different versions of the panel.

In conclusion, we report on the diagnostic rate among all samples sent for congenital limb malformations to a clinical reference laboratory over a 3-year period of time. In more than 300 individuals screened for 52 heritable limb malformation genes/regulatory regions, 37.2% had a pathogenic or probably pathogenic variant, of which 52 were novel variants. Variants of uncertain significance in highly candidate genes were identified in 6% of patients. Fifty-seven percent of the patients (200/352) were tested negative (no variant identified), suggesting other genomic regions or physio-pathological mechanisms involved.

### **Acknowledgements**

We gratefully acknowledge the following: Cindy Crunelle, Marthe Cochard and the bioinformatics team of Lille University Hospital for technical support; Anaïs Covain and Laurence Bellengier for administrative support; Pr. Nigel Quayle for manuscript editing. Also, we thank all the clinicians, patients and families who contributed to this work.

The authors have no conflict of interest to declare.

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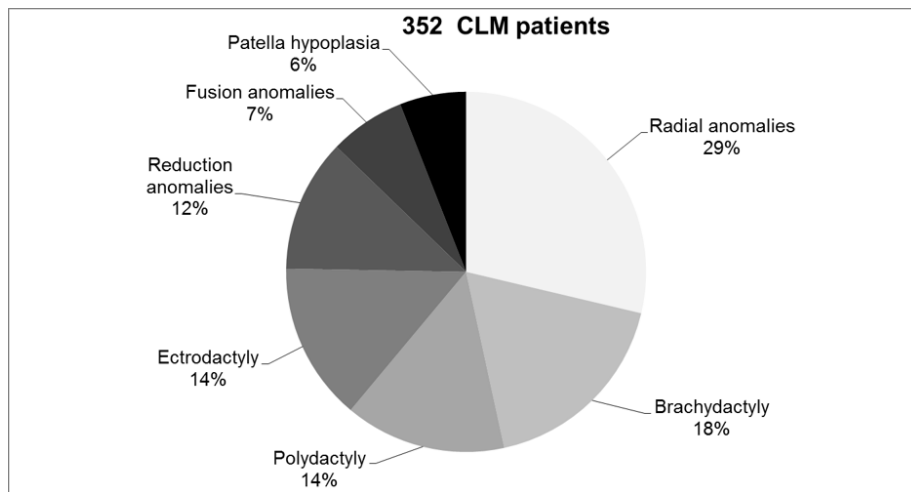
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The data that support the findings of this study are openly available in ClinVar database (<https://www.ncbi.nlm.nih.gov/clinvar/>) under the submission ID SUB5823379.

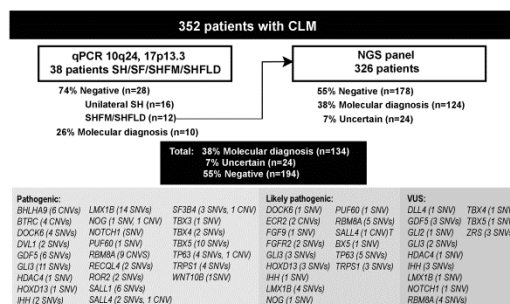


## Figures

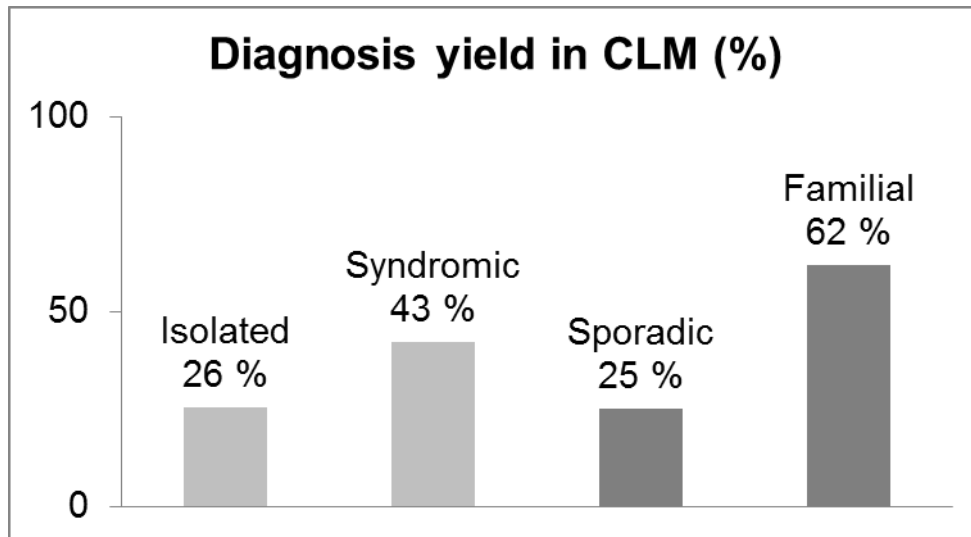
**Figure 1:** Description of the patient series by phenotype groups.



**Figure 2:** Summary of molecular analyses performed, diagnostic yield and variants identified in our patient series.



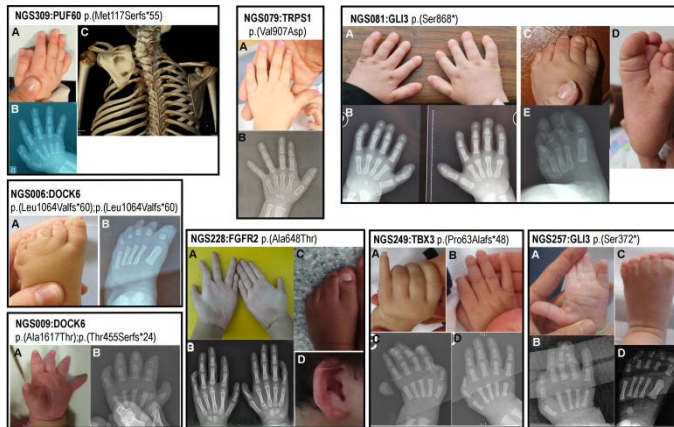
**Figure 3:** Diagnosis yield in our series of patients affected with CLM. Percentage of patients for whom a pathogenic or likely pathogenic variant has been identified, depending on the phenotype (isolated or syndromic CLM) and the pedigree (sporadic or familial case).



**Figure 4:** Pictures and radiographs of selected patients affected with congenital limb malformations.

NGS309: A,B: Picture and X-ray of the right hand showing duplicated thumb. C: 3D-scan showing left Sprengel anomaly with fusion of cervical vertebrae. NGS079: A,B: Picture and X-rays of the left hand showing type E brachydactyly. NGS081: A,B: Picture and X-rays of hands showing short middle and distal phalanges associated with bilateral triphalangeal thumbs. C,D,E: Pictures and X-rays of the left foot showing large hallux, clinodactyly of toes and a median plantar crease. NGS006: A, B: Picture and X-rays of the left foot showing terminal transverse defects of the toes. NGS009: A, B: Picture and X-rays of the left hand showing terminal transverse defects of the fingers. NGS228: A,B: Picture and X-rays of hands showing bilateral hypoplastic thumbs and 5<sup>th</sup> fingers clinodactyly. C: 2-3 syndactyly of toes on the left foot. D: Small dysplastic right ear. NGS249: A-D: Pictures and X-rays of hands showing a left postaxial polydactyly, associated with synostosis of the middle and

distal phalanges of the 5<sup>th</sup> fingers responsible for a limited flexion. Note volar nail of the left 5<sup>th</sup> finger. NGS257: A,B: Picture and X-rays of the hands showing 2-3-4 syndactyly of fingers. C,D: Picture and X-rays of the left foot showing 1-2-3-4 syndactyly of toes and a large hallux.



Cas e ID	Phenoty pic group	Phenot ypic subgro up	Is ol / S d	F am / S por	Gene / regul atory elem ent	Zygos ity	Va riant typ e	cDNA/gDNA change	Protein change	A C M G cl as s	References (PMID#)
qP CR 002	Ectrodac tily	SH/SF	Is ol	S por	<i>BHLH A9</i>	HTZ	CN V	g.?-?insNC_000017.10:(?_1174068)_(1174831_?)	p.(?)	5	23202277, 23790188
qP CR 012	Ectrodac tily	SHFLD	Is ol	F am	<i>BHLH A9</i>	HTZ	CN V	g.?-?insNC_000017.10:(?_1174068)_(1174831_?)	p.(?)	5	23202277, 23790188
qP CR 028	Ectrodac tily	SHFLD	Is ol	S por	<i>BHLH A9</i>	HTZ	CN V	g.?-?insNC_000017.10:(?_1174068)_(1174831_?)	p.(?)	5	23202277, 23790188
qP CR 037	Ectrodac tily	SH/SF	Is ol	S por	<i>BHLH A9</i>	HTZ	CN V	g.?-?insNC_000017.10:(?_1174068)_(1174831_?)	p.(?)	5	23202277, 23790188
qP CR 039	Ectrodac tily	SH/SF	Is ol	S por	<i>BHLH A9</i>	HTZ	CN V	g.?-?insNC_000017.10:(?_1174068)_(1174831_?)	p.(?)	5	23202277, 23790188
qP CR 042	Ectrodac tily	SH/SF	Is ol	F am	<i>BHLH A9</i>	HTZ	CN V	g.?-?insNC_000017.10:(?_1174068)_(1174831_?)	p.(?)	5	23202277, 23790188

qP CR 009	Ectrodac tyly	SHFM	Is ol	F a m	<i>BTRC</i>	HTZ	CN V	g.?_?insNC_000010.10: (?_103281432)_(103313 499_?)	p.(?)	5	30622331
qP CR 015	Ectrodac tyly	SHFM	Is ol	N D	<i>BTRC</i>	HTZ	CN V	g.?_?insNC_000010.10: (?_103281432)_(103313 499_?)	p.(?)	5	30622331
qP CR 020	Ectrodac tyly	SHFM	Is ol	F a m	<i>BTRC</i>	HTZ	CN V	g.?_?insNC_000010.10: (?_103281432)_(103313 499_?)	p.(?)	5	30622331
qP CR 023	Ectrodac tyly	SHFM	Is ol	F a m	<i>BTRC</i>	HTZ	CN V	g.?_?insNC_000010.10: (?_103281432)_(103313 499_?)	p.(?)	5	30622331
NG S00 6	Reductio n anomalie s	Adams- Oliver	S d	S p or	<i>DOC K6</i>	HMZ	SN V	c.[3190_3191delCT]	p.(Leu10 64Valfs* 60)	5	25091416
NG S00 9	Reductio n anomalie s	Adams- Oliver	S d	S p or	<i>DOC K6</i>	Comp ound HTZ	SN V	c.[4849G>A]	p.[(Ala16 17Thr)]	4	This report
								c.[1362_1365del]	p.[(Thr45 5Serfs*2 4)]	5	21820096, 29620724
NG S01 4	Reductio n anomalie s	Adams- Oliver	S d	S p or	<i>DOC K6</i>	HMZ	SN V	c.[1833-1G>T]	p.[(?)]	5	This report
NG S27 2	Brachyd actyly	Robino w	S d	S p or	<i>DVL1</i>	HTZ	SN V	c.1640-1G>A	p.(?)	5	This report
NG S27 3	Brachyd actyly	Robino w	S d	S p or	<i>DVL1</i>	HTZ	SN V	c.1505delA	p.(His50 2Profs*1 47)	5	This report
NG S02 7	Brachyd actyly	BDA	Is ol	F a m	<i>ECR2 (BMP 2)</i>	HTZ	CN V	g.?_?insNC_000020.10: (?_6864065)_(6865794_ ?)	p.(?)	4	21357617
NG S05 0	Brachyd actyly	BDC	Is ol	F a m	<i>ECR2 (BMP 2)</i>	HTZ	CN V	g.?_?insNC_000020.10: (?_6861756)_(6864880_ ?)	p.(?)	4	21357617
NG S28 3	Fusion anomalie s	Multiple synosto sis	S d	S p or	<i>FGF9</i>	HTZ	SN V	c.184A>G	p.(Arg62 Gly)	4	28730625
NG S28 2	Fusion anomalie s	Syndact yly	S d	S p or	<i>FGFR 2</i>	HTZ	SN V	c.755C>G	p.(Ser25 2Trp)	4	7719344,865
NG S22 8	Radial anomalie s	Okiihiro	S d	F a m	<i>FGFR 2</i>	HTZ	SN V	c.1942G>A	p.(Ala64 8Thr)	4	16501574,24
NG S04 9	Brachyd actyly	BDC	Is ol	F a m	<i>GDF5</i>	HTZ	SN V	c.989_997delinsGCCGC GCC	p.(Asp33 0Glyfs*1 23)	5	This report
NG S05 2	Brachyd actyly	BDC	Is ol	F a m	<i>GDF5</i>	HTZ	SN V	c.498dupC	p.(Ile167 Hisfs*18)	5	15173244

NG S054	Brachyactyly	BDC	Isol	Fam	<i>GDF5</i>	HTZ	SNV	c.549delT	p.(Asp184Thrfs*9)	5	This report
NG S056	Brachyactyly	BDC	Isol	Spor	<i>GDF5</i>	HTZ	SNV	c.1471G>T	p.(Glu491*)	5	16892395
NG S057	Brachyactyly	BDC	Isol	Fam	<i>GDF5</i>	HTZ	SNV	c.157dupC	p.(Leu53Profs*41)	5	12357473, 27577507
NG S060	Brachyactyly	BDC	Isol	Spor	<i>GDF5</i>	HTZ	SNV	c.1108dupT	p.(Tyr370Leufs*2)	5	This report
NG S081	Brachyactyly	Unclassified	Sd	Spor	<i>GLI3</i>	HTZ	SNV	c.2603C>A	p.(Ser868*)	5	This report
NG S257	Fusion anomalies	Syndactyly	Isol	Fam	<i>GLI3</i>	HTZ	SNV	c.1115C>A	p.(Ser372*)	5	This report
NG S063	Polydactyly	Mixed	Isol	Fam	<i>GLI3</i>	HTZ	SNV	c.2867delG	p.(Arg956Profs*47)	5	This report
NG S234	Polydactyly	Mixed	Isol	Fam	<i>GLI3</i>	HTZ	SNV	c.444C>A	p.(Tyr148*)	5	24736735
NG S235	Polydactyly	Mixed	Sd	Fam	<i>GLI3</i>	HTZ	SNV	c.1798delA	p.(Thr600Argfs*29)	5	This report
NG S238	Polydactyly	Mixed	Isol	Fam	<i>GLI3</i>	HTZ	SNV	c.1999C>T	p.(Arg667*)	5	25525159
NG S240	Polydactyly	Mixed	Isol	Fam	<i>GLI3</i>	HTZ	SNV	c.919C>T	p.(Gln307*)	5	This report
NG S243	Polydactyly	Mixed	Sd	Spor	<i>GLI3</i>	HTZ	SNV	c.1698C>G	p.(His566Gln)	4	This report
NG S246	Polydactyly	Postaxial	Sd	Spor	<i>GLI3</i>	HTZ	SNV	c.2211delT	p.(Ser737Argfs*2)	5	This report
NG S251	Polydactyly	Postaxial	Isol	Spor	<i>GLI3</i>	HTZ	SNV	c.1622C>T	p.(Thr541Met)	4	This report
NG S253	Polydactyly	Mixed	Sd	Fam	<i>GLI3</i>	HTZ	SNV	c.1274_1275delGC	p.(Ser425Asnfs*3)	5	This report
NG S260	Polydactyly	Preaxial	Sd	Spor	<i>GLI3</i>	HTZ	SNV	c.1622C>T	p.(Thr541Met)	4	This report
NG S262	Polydactyly	Preaxial	Isol	Spor	<i>GLI3</i>	HTZ	SNV	c.1687_1694del	p.(Leu563Thrfs*14)	5	This report

NG S315	Polydactyly	Postaxial	Sd	Spor	<i>GLI3</i>	HTZ	SNV	c.2799C>A	p.(Tyr933*)	5	15739154
NG S070	Brachydactyly	BDE	Sd	Spor	<i>HDA C4</i>	HTZ	SNV	c.339+1G>A	p.(?)	5	This report
NG S039	Brachydactyly	BDA	Isol	Fam	<i>HOX D13</i>	HTZ	SNV	c.917G>A	p.(Arg306Gln)	4	22374128, 24789103
NG S075	Polydactyly	Synpolydactyly	Isol	Fam	<i>HOX D13</i>	HTZ	SNV	c.186_212dup	p.(Ala65_Ala71dup)	4	This report
NG S076	Polydactyly	Synpolydactyly	Isol	Spor	<i>HOX D13</i>	HTZ	SNV	c.186_212dup	p.(Ala63_Ala71dup)	5	This report
NG S184	Polydactyly	Synpolydactyly	Isol	Fam	<i>HOX D13</i>	HTZ	SNV	c.186_212dup	p.(Ala63_Ala71dup)	4	This report
NG S020	Brachydactyly	BDA	Isol	Spor	<i>IHH</i>	HTZ	SNV	c.391G>A	p.(Glu131Lys)	5	11455389, 21537345
NG S026	Brachydactyly	BDA	Isol	Fam	<i>IHH</i>	HTZ	SNV	c.298G>A	p.(Asp100Asn)	4	12384778
NG S029	Brachydactyly	BDA	Isol	Spor	<i>IHH</i>	HTZ	SNV	c.391G>A	p.(Glu131Lys)	5	11455389, 21537345
NG S153	Patella hypoplasia	Nail-Patella	Sd	ND	<i>LMX1 B</i>	HTZ	SNV	c.782G>C	p.(Arg261Pro)	4	22574102
NG S154	Patella hypoplasia	Nail-Patella	Sd	Spor	<i>LMX1 B</i>	HTZ	SNV	c.755T>C	p.(Leu252Pro)	5	10571942
NG S157	Patella hypoplasia	Nail-Patella	Sd	Fam	<i>LMX1 B</i>	HTZ	SNV	c.317A>G	p.(Asp106Gly)	4	10571942
NG S161	Patella hypoplasia	Nail-Patella	Sd	Fam	<i>LMX1 B</i>	HTZ	SNV	c.175T>C	p.(Cys59Arg)	5	10571942
NG S163	Patella hypoplasia	Nail-Patella	Sd	Spor	<i>LMX1 B</i>	HTZ	SNV	c.217G>T	p.(Glu73*)	5	This report
NG S167	Patella hypoplasia	Nail-Patella	Sd	Fam	<i>LMX1 B</i>	HTZ	SNV	c.668G>A	p.(Arg233Gln)	5	This report
NG S169	Patella hypoplasia	Nail-Patella	Sd	Fam	<i>LMX1 B</i>	HTZ	SNV	c.661C>T	p.(Arg221*)	5	9590287, 24720768
NG S170	Patella hypoplasia	Nail-Patella	Sd	Fam	<i>LMX1 B</i>	HTZ	SNV	c.741+1G>A	p.(?)	5	9837817

NG S173	Patella hypoplasia	Nail-Patella	Sd	ND	<i>LMX1B</i>	HTZ	SNV	c.736C>T	p.(Arg246*)	5	9837817
NG S174	Patella hypoplasia	Nail-Patella	Sd	ND	<i>LMX1B</i>	HTZ	SNV	c.755T>C	p.(Leu252Pro)	4	10571942
NG S175	Patella hypoplasia	Nail-Patella	Sd	Spor	<i>LMX1B</i>	HTZ	SNV	c.139+2dupT	p.(?)	4	This report
NG S176	Patella hypoplasia	Nail-Patella	Sd	Fam	<i>LMX1B</i>	HTZ	SNV	c.661C>T	p.(Arg221*)	5	9590287, 24720768
NG S182	Patella hypoplasia	Nail-Patella	Sd	Fam	<i>LMX1B</i>	HTZ	SNV	c.661C>T	p.(Arg221*)	5	9590287, 24720768
NG S190	Patella hypoplasia	Nail-Patella	Sd	Spor	<i>LMX1B</i>	HTZ	SNV	c.668G>A	p.(Arg223Gln)	5	This report
NG S194	Patella hypoplasia	Nail-Patella	Sd	Fam	<i>LMX1B</i>	HTZ	SNV	c.668G>A	p.(Arg223Gln)	5	This report
NG S199	Patella hypoplasia	Nail-Patella	Sd	Fam	<i>LMX1B</i>	HTZ	SNV	c.303_304delGT	p.(Tyr102Leufs*45)	5	9618165
NG S214	Patella hypoplasia	Nail-Patella	Sd	Fam	<i>LMX1B</i>	HTZ	SNV	c.302_303delTG	p.(Tyr102Leufs*45)	5	This report
NG S215	Patella hypoplasia	Nail-Patella	Sd	Spor	<i>LMX1B</i>	HTZ	SNV	c.220T>C	p.(Ser74Pro)	5	This report
NG S287	Fusion anomalies	Nail-Patella	Sd	Spor	<i>NOG</i>	HTZ	SNV	c.253delG	p.(Glu85Argfs*39)	5	This report
NG S291	Fusion anomalies	Nail-Patella	Sd	Fam	<i>NOG</i>	HTZ	CNV	c.(?_1)_(699_?)del	p.(?)	5	This report
NG S296	Fusion anomalies	Nail-Patella	Sd	Fam	<i>NOG</i>	HTZ	SNV	c.613T>G	p.(Trp205Gly)	4	This report
NG S003	Reduction anomalies	Adams-Oliver	Sd	Spor	<i>NOTCH1</i>	HTZ	SNV	c.1941dupC	p.(Ser648Glnfs*20)	5	This report
NG S226	Radial anomalies	Okiihiro	Sd	Spor	<i>PUF60</i>	HTZ	SNV	c.407_410delTCTA	p.(Ile136Thrfs*31)	5	This report
NG S309	Radial anomalies	Townes-Brocks	Sd	Spor	<i>PUF60</i>	HTZ	SNV	c.349-13T>A	p.(Met117Serfs*55)	4	This report
NG S101	Radial anomalies	TAR	Sd	Spor	<i>RBM8A</i>	HTZ	CNV	c.(?_206-58)_(479+40_?)del	p.(?)	5	17236129

NG S150	Radial anomalies	TAR	Sd	Fam	<i>RBM8A</i>	Hemiz	CNV	c.(?_206-58)_(479+40_?)del	p.(?)	5	17236129
NG S208	Radial anomalies	TAR	Sd	Fam	<i>RBM8A</i>	Hemiz	CNV	c.(?_206-58)_(479+40_?)del	p.(?)	5	17236129
NG S212	Radial anomalies	TAR	Sd	Spor	<i>RBM8A</i>	Hemiz	CNV	c.(?_206-58)_(479+40_?)del	p.(?)	5	17236129
NG S252	Radial anomalies	TAR	Sd	Fam	<i>RBM8A</i>	Hemiz	SNV	c.[_21G>A]	p.(?)	4	22366785, 22366785
							CNV	c.(?_206-58)_(479+40_?)del	p.(?)	5	17236129
NG S256	Radial anomalies	TAR	Sd	Spor	<i>RBM8A</i>	Hemiz	SNV	c.[_21G>A]	p.(?)	4	22366785, 22366785
							CNV	c.(?_206-58)_(479+40_?)del	p.(?)	5	17236129
NG S263	Radial anomalies	TAR	Sd	Spor	<i>RBM8A</i>	Hemiz	SNV	c.[_21G>A]	p.(?)	4	22366785, 22366785
NG S277	Radial anomalies	TAR	Sd	Spor	<i>RBM8A</i>	HTZ	CNV	c.(?_206-58)_(479+40_?)del	p.(?)	5	17236129
NG S297	Radial anomalies	TAR	Sd	Spor	<i>RBM8A</i>	Hemiz	SNV	c.[_21G>A]	p.(?)	4	22366785, 22366785
							CNV	c.(?_206-58)_(479+40_?)del	p.(?)	5	17236129
NG S300	Radial anomalies	TAR	Sd	Fam	<i>RBM8A</i>	Hemiz	SNV	c.[_21G>A]	p.(?)	4	22366785, 22366785
							CNV	c.(?_206-58)_(479+40_?)del	p.(?)	5	17236129
NG S271	Radial anomalies	RECQL4	Sd	Spor	<i>RECQL4</i>	Compound HTZ	SNV	c.[3_4delinsTT]	p.(?)	5	This report
							CNV	c.[2590C>T]	p.(Gln864*)	5	24635570
NG S043	Brachyactyly	BDB	Isol	Fam	<i>ROR2</i>	HTZ	SNV	c.1394_1395delTC	p.(Leu465Glnfs*59)	5	This report
NG S047	Brachyactyly	BDB	Isol	Spor	<i>ROR2</i>	HTZ	SNV	c.1397_1398delAA	p.(Lys466Argfs*58)	5	This report
NG S302	Radial anomalies	Townes-Brocks	Sd	Fam	<i>SALL1</i>	HTZ	SNV	c.1148delT	p.(Leu383Tyrfs*2)	5	9973281
NG S304	Radial anomalies	Townes-Brocks	Sd	Fam	<i>SALL1</i>	HTZ	SNV	c.3034delT	p.(Cys1012Valfs*33)	5	This report
NG S30	Radial anomalies	Townes-Brocks	Sd	Fam	<i>SALL1</i>	HTZ	SNV	c.3168delG	p.(Pro1057His*19)	5	This report



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NG S307	Radial anomalies	Townes-Brocks	Sd	Fam	SALL1	HTZ	SNV	c.2999delA	p.(Asn100Thrfs*45)	5	This report		
NG S314	Radial anomalies	Townes-Brocks	Sd	Spor	SALL1	HTZ	SNV	c.902dupA	p.(Ser302Glufs*10)	5	This report		
NG S320	Radial anomalies	Townes-Brocks	Sd	Spor	SALL1	HTZ	SNV	c.824T>G	p.(Leu275*)	5	29110636		
NG S217	Radial anomalies	Okiihiro	Sd	Fam	SALL4	HTZ	SNV	c.1242_1245del	p.(Cys415Valfs*20)	5	This report		
NG S220	Radial anomalies	Okiihiro	Sd	Spor	SALL4	HTZ	CNV	c.(130+1_131-1)(*1548_?)del	p.(?)	5	20301547		
NG S223	Radial anomalies	Okiihiro	Sd	Spor	SALL4	HTZ	SNV	c.1717C>T	p.(Arg573*)	5	This report		
NG S301	Radial anomalies	Okiihiro	Sd	Fam	SALL4	HTZ	CNV	c.(?_2743)_(3162_?)del	p.(?)	4	20301547		
NG S143	Radial anomalies	Nager	Sd	Spor	SF3B4	HTZ	SNV	c.1A>T	p.(Met1?)	5	This report		
NG S144	Radial anomalies	Nager	Sd	Spor	SF3B4	HTZ	SNV	c.827dupC	p.(Ser277Ilefs*209)	5	22541558		
NG S151	Radial anomalies	Nager	Sd	Spor	SF3B4	HTZ	SNV	c.1147dupC	p.(His383Profs*?)	5	22541558		
NG S324	Radial anomalies	Nager	Sd	Spor	SF3B4	HTZ	CNV	c.(?_-69)_(1088-36_?)del	p.(?)	5	26679067		
NG S249	Polydactyly	Preaxial	Sd	Fam	TBX3	HTZ	SNV	c.185dupA	p.(Pro63Alafs*48)	5	This report		
NG S198	Patella hypoplasia	Small Patella	Isol	Fam	TBX4	HTZ	SNV	c.781C>T	p.(Arg261*)	5	This report		
NG S281	Patella hypoplasia	Small Patella	Sd	Spor	TBX4	HTZ	SNV	c.781C>T	p.(Arg261*)	5	This report		
NG S117	Radial anomalies	Holt-Oram	Sd	Fam	TBX5	HTZ	SNV	c.709C>T	p.(Arg237Trp)	5	10077612, 12499378, 19648116		
NG S120	Radial anomalies	Holt-Oram	Sd	Fam	TBX5	HTZ	SNV	c.668C>T	p.(Thr223Met)	5	12789647		
NG S12	Radial anomalies	Holt-Oram	Sd	Spor	TBX5	HTZ	SNV	c.436delG	p.(Ala146Argfs*4)	5	This report		

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NG S124	Radial anomalies	Holt-Oram	Sd	Fam	<i>TBX5</i>	HTZ	SNV	c.342C>A	p.(Tyr114*)	5	16183809, 25525159
NG S129	Radial anomalies	Holt-Oram	Sd	Spor	<i>TBX5</i>	HTZ	SNV	c.1319delC	p.(Ala440Valfs*142)	5	This report
NG S131	Radial anomalies	Holt-Oram	Sd	Fam	<i>TBX5</i>	HTZ	SNV	c.313_325dup	p.(Ala109Glyfs*15)	5	This report
NG S132	Radial anomalies	Holt-Oram	Sd	Spor	<i>TBX5</i>	HTZ	SNV	c.798delA	p.(Val267Trpfs*127)	5	12789647
NG S133	Radial anomalies	Holt-Oram	Sd	Fam	<i>TBX5</i>	HTZ	SNV	c.(755+1_756-1)_(*1602-?)del	p.(?)	4	This report
NG S135	Radial anomalies	Holt-Oram	Sd	Spor	<i>TBX5</i>	HTZ	SNV	c.593dup	p.(Asn198Lysfs*11)	5	8988164
NG S136	Radial anomalies	Holt-Oram	Sd	Fam	<i>TBX5</i>	HTZ	SNV	c.1011dup	p.(Tyr338Leufs*2)	5	This report
NG S142	Radial anomalies	Holt-Oram	Isol	Fam	<i>TBX5</i>	HTZ	SNV	c.710G>A	p.(Arg237Gln)	5	8988165, 12499378, 19648116
NG S089	Ectrodactyly	EEC	Sd	Spor	<i>TP63</i>	HTZ	SNV	c.953G>A	p.(Arg318His)	5	10535733, 18626511
NG S090	Ectrodactyly	EEC	Sd	Spor	<i>TP63</i>	HTZ	SNV	c.727C>T	p.(Arg243Trp)	5	10535733, 18626511
NG S097	Ectrodactyly	EEC	Sd	Fam	<i>TP63</i>	HTZ	SNV	c.692A>G	p.(Tyr231Cys)	4	12037717, 28777841
NG S109	Ectrodactyly	EEC	Sd	Spor	<i>TP63</i>	HTZ	SNV	c.727C>T	p.(Arg243Trp)	4	10535733, 18626511
NG S112	Ectrodactyly	EEC	Sd	Spor	<i>TP63</i>	HTZ	SNV	c.955C>T	p.(Arg319Cys)	5	10839977, 12161593, 18626511
NG S113	Ectrodactyly	EEC	Sd	Spor	<i>TP63</i>	HTZ	SNV	c.1049G>A	p.(Arg350Lys)	4	This report
NG S114	Ectrodactyly	EEC	Sd	Spor	<i>TP63</i>	HTZ	SNV	c.727C>T	p.(Arg243Trp)	5	10535733, 18626511
NG S116	Ectrodactyly	EEC	Sd	Spor	<i>TP63</i>	HTZ	SNV	c.953G>A	p.(Arg318His)	4	10535733, 18626511
NG S27	Ectrodactyly	SHFM	Sd	Spor	<i>TP63</i>	HTZ	CNV	c.(?_1653-65)_(1873_?)del	p.(?)	5	24163146

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NG S279	Ectrodactyly	SHFM	Isol	Spor	<i>TP63</i>	HTZ	SNV	c.692A>G	p.(Tyr231Cys)	4	12037717, 28777841
NG S079	Brachydactyly	BDE	Sd	Fam	<i>TRPS1</i>	HTZ	SNV	c.2720T>A	p.(Val907Asp)	4	11112658
NG S091	Brachydactyly	TRPS	Sd	Spor	<i>TRPS1</i>	HTZ	SNV	c.2761C>G	p.(Arg921Gly)	5	25792522, 28170084
NG S093	Brachydactyly	TRPS	Sd	ND	<i>TRPS1</i>	HTZ	SNV	c.2789G>C	p.(Cys930Ser)	4	This report
NG S195	Brachydactyly	TRPS	Sd	Spor	<i>TRPS1</i>	HTZ	SNV	c.2705_2706dupGT	p.(Arg903Valfs*12)	5	This report
NG S321	Brachydactyly	TRPS	Sd	Spor	<i>TRPS1</i>	HTZ	SNV	c.2894G>A	p.(Arg965His)	5	14560312
NG S323	Brachydactyly	TRPS	Sd	ND	<i>TRPS1</i>	HTZ	SNV	c.2894G>A	p.(Arg965His)	5	14560312
NG S325	Brachydactyly	TRPS	Isol	Spor	<i>TRPS1</i>	HTZ	SNV	c.2732A>G	p.(Asn911Ser)	4	25792522
NG S276	Ectrodactyly	SH/SF	Isol	Fam	<i>WNT10B</i>	HTZ	SNV	c.343C>T	p.(Arg115*)	5	This report

**Table 1:** Likely pathogenic or pathogenic variants identified in our patient series. . Isol: isolated; Sd: syndromic; Fam: familial; Spor: sporadic; ACMG: American College of Medical Genetics and Genomics; SNV: single nucleotide variant; CNV: copy-number variation; HMZ: homozygous; HTZ: heterozygous; HemiZ: Hemizygous; ND: not determined; SH: split hand; SF: split foot; SHFM: split hand/foot malformation; SHFLD: split hand/foot malformation with long-bone deficiency; TAR: thrombocytopenia-absent radius; TRPS: Tricho-rhino-phalangeal; EEC: ectrodactyly-ectodermal dysplasia-cleft lip/palate; BDA, BDB, BDC, BDE: brachydactyly type A, B, C or E.

Phenotype	Patients (Nb)	%	Molecular diagnoses (Nb)	Diagnostic yield (%)	Genes (patients Nb)
<b>Radial anomalies</b>	<b>101</b>	<b>28,7</b>	<b>33</b>	<b>32,7</b>	
Holt-Oram Syndrome	34	9,7	11	32,4	<i>TBX5</i> (11)
Okhiro Syndrome	20	5,7	6	30,0	<i>SALL4</i> (4) <i>PUF60</i> (1) <i>FGFR2</i> (1)
Townes-Brocks Syndrome	20	5,7	7	35,0	<i>SALL1</i> (6) <i>PUF60</i> (1)
TAR Syndrome	11	3,1	4	36,4	<i>RBM8A</i> (4)
Nager Syndrome	8	2,3	4	50,0	<i>SF3B4</i> (4)
<i>RECQL4</i> disorders	3	0,9	1	33,3	<i>RECQL4</i> (1)
Unclassified	5	1,4	0	0,0	
<b>Ectrodactyly</b>	<b>50</b>	<b>14,2</b>	<b>20</b>	<b>40,0</b>	
SH/SF	24	6,8	4	16,7	<i>BHLHA9</i> (4)
SHFLD	4	1,1	2	50,0	<i>BHLHA9</i> (2)
SHFM	13	3,7	6	46,2	<i>TP63</i> (2) <i>BTRC</i> (4)
EEC	9	2,6	8	88,9	<i>TP63</i> (8)
<b>Brachydactyly</b>	<b>63</b>	<b>17,9</b>	<b>25</b>	<b>39,7</b>	
BDE	19	5,4	2	10,5	<i>HDAC4</i> (1) <i>TRPS1</i> (1)
BDA	11	3,1	5	45,5	<i>IHH</i> (3) <i>HOXD13</i> (1) <i>ECR2</i> (1)
BDC	9	2,6	7	77,8	<i>GDF5</i> (6) <i>ECR2</i> (1)
BDB	5	1,4	2	40,0	<i>ROR2</i> (2)
Tricho-rhino-phalangeal Syndrome	9	2,6	6	66,7	<i>TRPS1</i> (6)
Robinow Syndrome	2	0,6	2	100,0	<i>DVL1</i> (2)
Unclassified	8	2,3	1	12,5	<i>GLI3</i> (1)
<b>Polydactyly</b>	<b>51</b>	<b>14,5</b>	<b>16</b>	<b>31,4</b>	

Preaxial	20	5,7	2	10,0	<i>GLI3</i> (2)
Postaxial	14	4,0	4	28,6	<i>GLI3</i> (3), <i>TBX3</i> (1)
Mixed	10	2,8	7	70,0	<i>GLI3</i> (7)
Synpolydactyly	6	1,7	3	50,0	<i>HOXD13</i> (3)
Unclassified	1	0,3	0	0,0	
<b>Reduction anomalies</b>	<b>42</b>	<b>11,9</b>	<b>4</b>	<b>9,5</b>	
Longitudinal	18	5,1	0	0,0	
Transversal	8	2,3	0	0,0	
Adams-Oliver Syndrome	9	2,6	4	44,4	<i>NOTCH1</i> (1) <i>DOCK6</i> (3)
Phocomelia-Amelia	3	0,9	0	0,0	
Unclassified	4	1,1	0	0,0	
<b>Fusion anomalies</b>	<b>24</b>	<b>6,8</b>	<b>6</b>	<b>25,0</b>	
Syndactyly	15	4,3	2	13,3	<i>FGFR2</i> (1) <i>GLI3</i> (1)
Multiple synostosis	9	2,6	4	44,4	<i>FGF9</i> (1) <i>NOG</i> (3)
<b>Patella hypoplasia</b>	<b>21</b>	<b>6,0</b>	<b>20</b>	<b>95,2</b>	
Nail Patella Syndrome	18	5,1	18	100,0	<i>LMX1B</i> (18)
Small Patella Syndrome	3	0,9	2	66,7	<i>TBX4</i> (2)
<b>Total</b>	<b>352</b>		<b>124</b>	<b>35,2</b>	

**Table 2:** Diagnostic yield in the patient series depending on the phenotype.

Nb: number; SH: split hand; SF: split foot; SHFM: split hand/foot malformation; SHFLD: split hand/foot malformation with long-bone deficiency; TAR: thrombocytopenia-absent radius; EEC: ectrodactyly-ectodermal dysplasia-cleft lip/palate; BDA, BDB, BDC, BDE: brachydactyly type A, B, C or E.