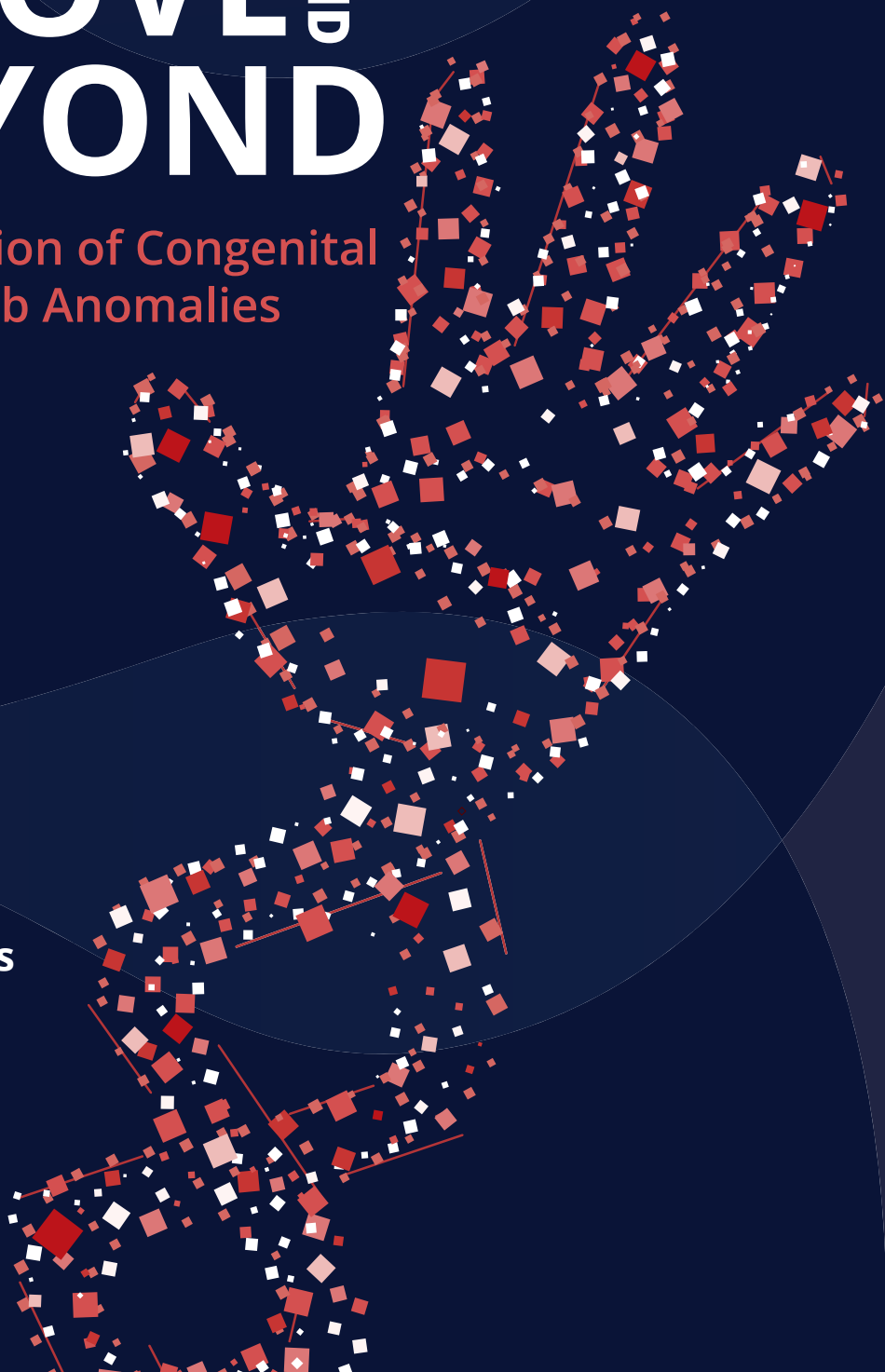
ABOVE AND BEYOND

Classification of Congenital Upper Limb Anomalies



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Above and Beyond

Classification of Congenital Upper Limb Anomalies

De onderste steen boven

classificatie van aangeboren afwijkingen van de bovenste extremiteiten

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PREFACE

The human hand and arm have always been fascinating to mankind. Doctors have been inflicted with the complexity of the anatomy, as illustrated by "The Anatomy Lesson" of Dr. Nicolaas Tulp (1632). Many believe that the synergistic development of hand and brain in evolution eventually distinguished mankind from other primates. Aristotle stated the hand is the "instrument of instruments" and is "for the body as the intellect is for the soul". Likewise, Kant has described the hand as "the man's outer brain". Developmental biologists have studied the exact mechanisms of hand development; however, many topics of debate remain.

Whereas the accepted sciences study general hand development, the study of individual hands often remains the domain of pseudo-sciences such as palmistry. In palmistry, one could state that the quote of Kant has been (over)-extrapolated, claiming the hand is "the mirror of the soul" and can predict character, life course, and disease by its composition.

This thesis does share the hypothesis that more can be learned from an individual's hand anatomy. However, instead of normally developed hands, this thesis focuses on patients with congenital upper limb anomalies (CULA). As this thesis will reveal, the in-depth study of the individual hand enables to go above and beyond the classification of the observed anomaly and enable physicians to learn more about etiology, associated anomalies, possible underlying syndromes, and their genetic substrate.

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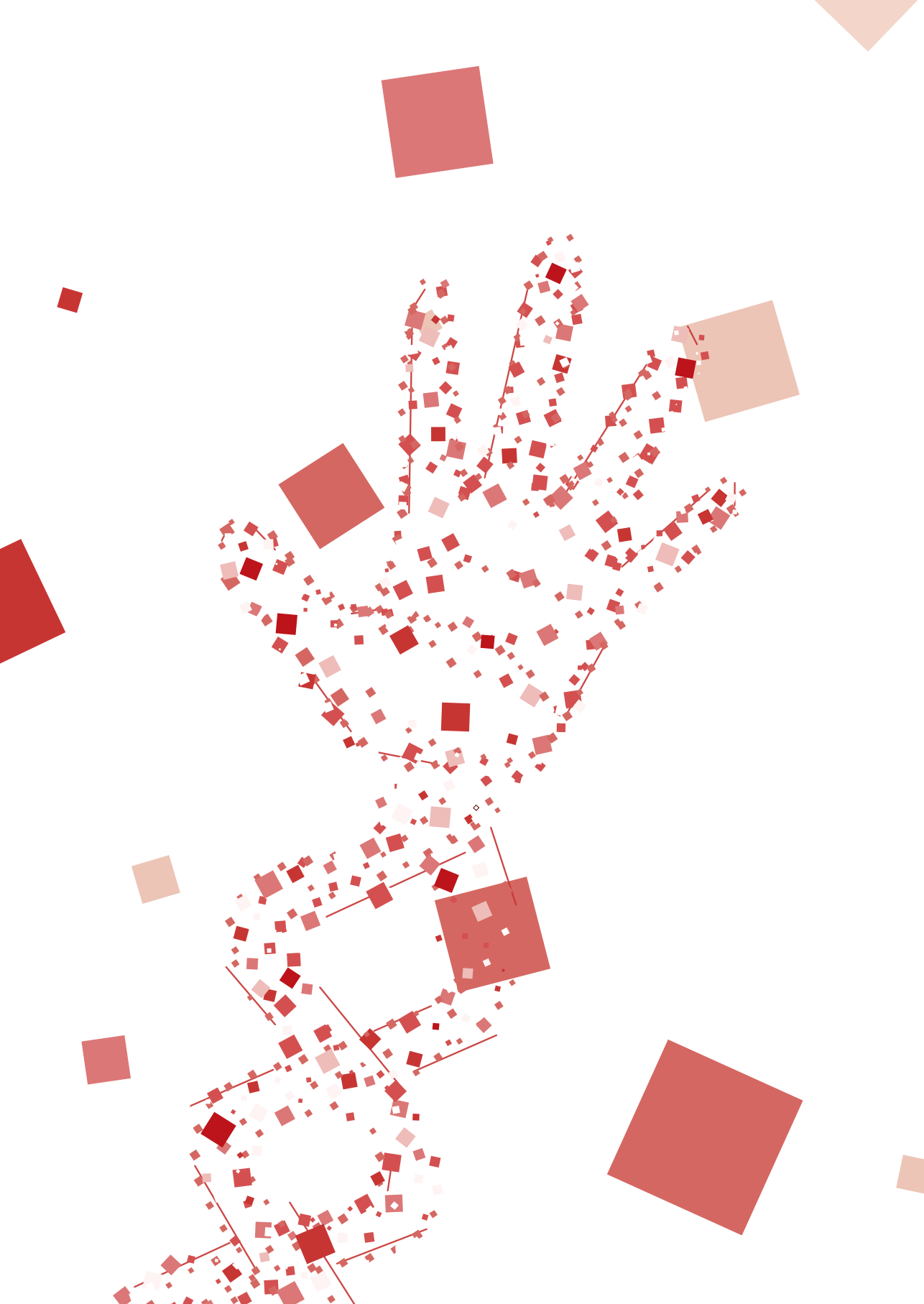

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General introduction

Congenital Upper Limb Anomalies

The term congenital upper limb anomaly (CULA) is used for any congenital anomaly observed in hand and upper limb anatomy. CULA occur in 1 in every 500 live births[1-3]. CULA exhibit a broad spectrum of phenotypic manifestations, and the prevalence of the individual anomalies vary from 1:2.000 to <1:1.000.000[2]. The most commonly observed CULA include radial and ulnar polydactyly, clinodactyly, camptodactyly, brachydactyly, and syndactyly. More disabling and rare anomalies include radial longitudinal deficiency, transverse reduction, or phocomelia (e.g., a well-known result of thalidomide use during pregnancy).

It is important to note that both the 'simple' and the more invalidating CULA can occur as an isolated malformation, combined with other anomalies or as a part of a syndrome or sequence. The prevalence of syndromes or recognized genetic conditions among those patients varies from 2%-34[1-3]. The prevalence of syndromes is not equally distributed among the different hand anomalies, with reported prevalence up to 100% in distinct phenotypes[2]. Therefore, physicians dealing with CULA should be aware of the varying risks of underlying syndromes for the distinct hand anomalies. Noted, children with evident syndromal characteristics will have visited many different doctors before referral for treatment of their hands, however, many associated anomalies can also remain sub-clinical till the moment of detailed physical examination or by additional studies such as an echocardiogram[4].

In clinical practice, classification schemes can be helpful to stratify risk estimation for such an underlying syndrome and protocolize decision making based on the observed class of hand anomalies in a patient[5,6]. Especially considering rare hand anomalies, using a classification system allows building local and international knowledge on the anomaly, its treatment, and related anomalies or underlying syndromes. Multiple classification systems have been proposed for this purpose, most of which have been subject to multiple revisions based on, e.g., improved insight into limb development[7-10]. The need to classify, the used classification schemes, and the strategy of classification is commonly debated and need a further introduction to appreciate the debate entirely.

Classification, classify-phobia, and the unclassifiable

In general, the act of classification is a natural attempt to relate a current observation to a spectrum of experienced events or observations. This natural urge to group in biology can be dated back to Aristotle in his Historia Animalium. In medical sciences, the first recognized classification system is the London Bills of Mortality which was developed in the 16th century. These data provided, and continue to provide, valuable insight into the plague epidemy and the factors that influenced the containment of the disease[11]. Similarly, in clinical practice, the continuous classification builds experience and eventually provides a decision-making framework. However, every clinician classifies using the determinants that they feel influence outcome. Thus, in literature, many experts try to shape their framework, often conflicting with those of other authors resulting in a tangle of classification systems describing overlapping patient groups. This crisscross of classification schemes can be illustrated by the anatomical classification of triphalangeal thumbs by hand surgeons versus the differentiation between opposable and non-opposable triphalangeal thumbs by geneticists[12,13]. The phobia expressed by clinicians, in my opinion, does therefore not reflect the act of personal classification but rather the tangle of classification schemes that do not provide the aimed frame of mind for the user. Similarly, "unclassifiable" reflects a case that cannot be put within a personal or universal spectrum of observations. Unclassifiable thus means a failed attempt to relate your observation to previous observations by yourself or the international community that developed a classification scheme.

The mismatch of personal versus universal classification schemes and unclassifiable cases are the base for the lump versus split discussion that is as old as the act of classification itself[14]. Those who lump share a broader view and assume the differences between cases are of inferior importance to their similarities. Splitters instead state that it is the slight difference between cases that determine the distinct differences in the outcomes of interest. In my opinion, the acknowledgment of your professional interests and experience in a given lump defines the necessity to split: the diagnosis "preaxial polydactyly" might be sufficient for pediatricians but is usually "split" in different preaxial polydactyly types using one of the modifications of the Wassel classification for hand surgeons or the classification by Temtamy and McKusick for geneticists[13,15]. Hand surgeons prefer a classification that relates to the surgical intervention, and the different modifications reflect the necessity to split based on experience with the anomalies and their surgical outcomes. Geneticists want to recognize patterns of malformations of both the hand and feet as different patterns have been related to different genetic substrates. Thus, raising the question: can you assume that your interests and experience reflect those of others in communication using a universal classification system?

A paralogue of this debate can be found in wine tasting:

At first, non-wine drinkers lump every wine into two groups: white or red wine. Using these poorly defined groups, a satisfactory rate of 2 out of 10 wines can be achieved. Looking to improve the wine tasting experience, you start to learn more about wines, thus slowly becoming a splitter. At first, you start ordering Chardonnay instead of White wine, and the score rises to 4 out of 10. Still not satisfied with this result, you start splitting the Chardonnay and learn to appreciate that besides taste, color, viscosity, wood aging, and nose of the wine help improve your score to 9 out of 10. Satisfied with yourself, you are now enjoying your new life as a wine connoisseur. Using just these five characteristics, you are more likely to find a wine that suits your needs. Like in surgery, the one time that the wine does not work out, this is probably due to the incompetence of the waiter, the wine tailor, or anyone other person involved with the process from grape to the glass.

Subsequently, looking to fill your wine cabinet with these near-perfect fit wines, you are visiting your local wine buyer and start your detailed description of your favorite wine using your five key characteristics. Bored, your local wine buyer summarizes your epistle with the 10-second phrase: "So, you are looking to buy a classical build chardonnay from the Languedoc region?". Using just three key characteristics, 2 of which are different from yours, this superior expert could lump your needs into one specific product. You go ahead and buy 10 Languedoc wines, drink them in the next few weeks and again reach a nine out of 10 score. There are differences between your split and the Languedoc-lump of the wine buyer, but the overlap results in the same 9/10 score. The question now is, does it matter how you achieved the common outcome? Can the less experienced wine taster adopt the lump strategy of the expert wine buyer to find a similar wine again? Or perhaps more important: what if the less experienced drinker adopts the expert technique for different wines? Do we still get a comparable outcome?

Classification of CULA

Now that we understand the different approaches in classification, one can imagine that multiple classification systems for hand anomalies have been proposed. The first attempt to classify the observed anomalies can be dated back to 1829 to Saint-Hilaire's *Propositions sur la monstruosité*[16]. However, the most well-known classification system for upper extremity surgeons has been the scheme initially proposed by Swanson in 1968[17]. After modifications by representatives of the American Society for Surgery of the Hand (ASSH), the IFSSH, and the International Society for Prosthetics and Orthotics (ISPO), it was accepted as the modified Swanson scheme or IFSSH classification in 1974[9].

The modified Swanson scheme, while also using then presumed etiologic groups, is primarily based on a morphological system and exists out of 7 major categories. Classification of a hand anomaly is done by assigning the observed (combination of) anomalies to one of 7 groups. Especially in the combination of anomalies, such as triphalangeal thumb and radial polydactyly, this results in a debate on the nature of the anomaly: should it be defined as a failure of differentiation or a duplication? Realistically, it is both, and it is the classification system itself that is lacking.

Table 1. Swanson classification

Group name	Example phenotypes / hand anomalies
Failure of formation	Thumb deficiency, Symbrachydactyly
Failure of differentiation	Syndactyly, Triphalangeal thumb, Poland syndrome
Duplication	Radial polydactyly, central polydactyly
Overgrowth	Macrodactyly
Undergrowth	Brachydactyly
Constriction ring	Constriction ring syndrome
Generalized abnormalities and syndromes	

Although still almost universally adopted, the IFSSH classification has generated much criticism ever since its introduction resulting in multiple modifications and new initiatives for better registration of combined anomalies[8,18-20]. Especially in the last decades, the developments in molecular biology, histochemistry, chromosomal analysis have shed additional light on the mechanisms behind the etiology of CULA. This improved knowledge has led to increased knowledge about developmental biology and newly emerged concepts in the pathogenesis of CULA, disregarding some of the Swanson/IFFSH classification group's etiological assumptions. Furthermore, the classifications and their concordant strategy lack the flexibility to express the full extent of the anomaly observed; thus, clinical registry forms were developed to be able to describe the clinical presentation.

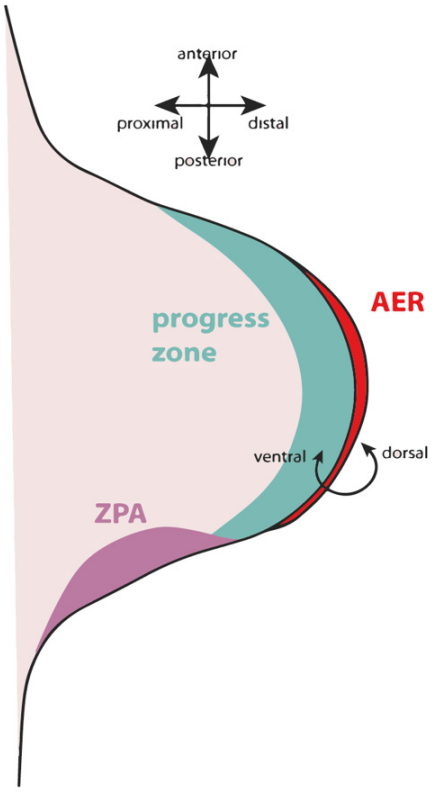
Therefore, in a multidisciplinary effort between developmental biologists and surgeons, Oberg, Feenstra, Manske, and Tonkin proposed a classification that incorporates our current comprehension of limb development in 2010: The OMT classification. Further additions and refinements took place in the years following its publication based upon the clinical and scientific use of the classification[2,5,7,20-24]. In response to the challenges with the Swanson scheme and recent developments, the IFSSH adopted the OMT classification for congenital hand anomalies in February 2014. To fully appreciate the differences between the Swanson and the OMT classification, embryology and its effect on the observed hand anomalies and syndromes should be discussed.

Embryology

Human limb development starts the 4th week (Carnegie stage 12) of conception[25-27]. A small limb bud is formed in this stage, which is an accumulation of mesoderm covered by a thin layer of ectoderm, located at somites 9-12. After the location of the limb buds is determined based on the local expression of transcription factors, the limb bud will elongate, and eventually a hand plate is formed. Proximally, by mesodermal condensation, a skeleton is formed called the stylopod (future shoulder and humerus), the zeugopod (future radius and ulna), and eventually the autopod (which will become the carpus and digits). In the autopod, apoptosis will separate the digits, eventually forming a 5-digit hand. The entire process takes approximately 23 days to complete. During these days, the outgrowth of the limb bud is regulated in different axes of development: the proximodistal axis, the dorsoventral axis, and the anteroposterior, or radioulnar axis.

Axes of development and limb outgrowth

The different axis-specific areas of proliferation can be specified using the apical ectodermal ridge (AER) as a reference (fig 1). The AER is located precisely on the dorsoventral border of the limb bud and is the key driving force of the proximo-distal axis of development. The proximo-distal axis is responsible for the 'length' growth of the limb, proliferative activity is found distally in the progress zone. The progress zone forms a layer of ectoderm that thickens over time and forms the AER. From this moment, the AER is considered the critical factor for proximo-distal outgrowth as experiments have illustrated that removing the AER leads to an arrest in proximo-distal outgrowth.



The zone of polarizing activity (ZPA) is the key signaling center for anteroposterior axis development. The anteroposterior axis is responsible for radio-ulnar differentiation of the limb. The ZPA is located underneath the AER, posteriorly located in the limb bud and was coincidentally discovered

by developmental biologist John Saunders. In his experiments for apoptosis, Saunders accidentally found that if a small region posteriorly located under the AER induced mirror image digit duplications. Subsequent studies have revealed that radio- ulnar differentiation is a Sonic Hedge Hog (SHH) mediated, dosage-dependent, process regulated by the ZPA. In normal development, the absence of SHH on the radial side induces the development of a thumb, whereas the presence of SHH induces digits to be formed.

Finally, the mesoderm ventral and dorsal of the AER are specific to the dorsoventral axis of development. Knockout of axis-specific genes results in a mouse model with footpads on both the volar and dorsal sides of the paw.

Classically, the axes of development are believed to have separate signaling centers with separate signaling pathways that control outgrowth for each specific axis. Over time, many interactions between the signaling pathways have been established, such as the AER/ZPA feedback loop (driven by FGF4/8 expression). These new interactions have fed a new hypothesis on digit identity formation based on the principles of the so-called Turing mechanism. This theory dictates that Turing waves dictate the digital and interdigital regions of the hand plates, similar to the theory of the origin of striped animals. However, mathematical modeling would suggest bifurcation of the distal phalanges if only the radioulnar axis is considered. Only if a proximo-distal gradient is applied, this bifurcation can be corrected for in modeling. Again, suggesting an interference of the different axes of development. Since these new theories are not widely accepted, this thesis will focus on the classical axis of development theorem.

Molecular control of limb outgrowth and clinical signs of disruption

Limb outgrowth starts with the determination of the position of the limb bud. For many anatomic structures, including the hand, the HOX transcription factor family (39 genes in four clusters A, B, C, D) is crucial[28]. Spatiotemporal expression of these different *HOX* genes is believed to orchestrate the overall body plan of the embryo. In the limb, mainly the *HOXA* and *HOXD* genes play a role in the development; deletions of both *HoxB* and *HoxD* do not show any abnormal phenotypes of the limbs in animal studies. The *Hoxa* and *Hoxd* genes are largely paralogs, and therefore their activity can grossly be considered in groups. In early limb bud development, mainly Hox groups 1-9 are active (thus, Hoxa 1-9 and Hoxd 1-9). These Hox groups can induce *Fgf10*, which is considered the driving force for AER development. As discussed, experimental removal of the AER results in an arrest of limb development, but interestingly this effect can be rescued by exogenous Fgfs. As a result, loss of group 9 Hox function results in an arrest in limb development

in the stylopod. Besides the hox genes, genes like *WNT3*, *TP63*, *TBX5*, and *SALL4* are required to establish the limb bud[29,30]. Interestingly, this can clinically be observed by loss of function due to mutation in *WNT3*, causing tetra-amelia[31,32]. Similarly, *TP63* encodes proteins required to maintain the AER, and mutations in *TP63* have been described to cause split hand/foot syndromes[33].

Similar to early limb bud initiation, later in proximo-distal axis development, *FGF10* and *FGF8* have an essential role in limb outgrowth[34]. Based on their induction, the limb bud will elongate and form a handplate. The handplate is the region in which most of the before mentioned AER activity and thus limb outgrowth can be found. During outgrowth, the before mentioned skeleton is formed: the stylopod, the zeugopod, and the autopod. The formation of these different sections is again influenced by the different *HOX* groups. Hox groups 8-10 are responsible for stylopod formation, whereas in the zeugopod and autopod, 11 through 13 are more active. These *Hox* genes both can induce Shh besides and partly instead of Fgf (groups 12 and 13)[28]. This change of regulatory effects is needed to induce the ZPA function for radio-ulnar axis development in the Zeugopod and Autopod. Again, the importance of these molecules can be illustrated by mutations in these genes. For example, rearrangements of the *Hoxd* cluster (10-13) can result in mesomelic dysplasia, with shortening of the forearms and digits, whereas isolated mutations in *HOXD13* only cause outgrowth problems of the digits[35,36].

In the dorsoventral axis, *WNT7a*, *LMX1b*, and *En-1* have a dominant effect[20,26,29]. In animal models, *Wnt7a* mutant limbs show both ventral and dorsal footpads[37]. In humans, multiple cases of patients with homozygous mutations in *WNT7a* have been reported[38]. These patients present with a limb/pelvis-hypoplasia/aplasia syndrome, with single bone forearms. However, these patients do not show the apparent loss of the dorsoventral axis as the mouse models might suggest. Similar findings have been described in man (palmar or circumferential nails); however, no genetic substrate was found for this hand anomaly, although 4q34 deletions have been suggested[39]. Mutations in *LMX1B* have been observed in Nail-Patella syndrome, a condition in which malformation of the nails, the elbows, and knees are observed[40,41]. A patella-like structure can be found in these patients fused to the distal head of the humerus and condyles that resemble femoral epicondyles of the knee. To my knowledge, no human diseases based on mutations of En-1 have been described.

Perhaps the most well-studied axis of development is the radio-ulnar axis. Based on local Hox expression, Shh production is induced[28,29,42]. Its production is mainly located on the posterior side of the limb bud, creating a gradient of Shh from the posterior to the anterior side of the limb bud. At the most anterior side of the limb bud, the thumb is formed in complete absence of Shh. However, this effect is not

solely caused by Shh but should also be attributed to its downstream transcription factors. A well-known example is GLI3, which has both an activator form (GLI3A) and a repressor form (GLI3R)[42-45]. Truncation of the GLI3 protein (which results in GLI3R) is an SHH-dependent process that is more active when lower levels of SHH are present. Mutations in GLI3 can cause polydactyly syndromes such as Polydactyly type 4, Greig syndrome and Pallister-Hall syndrome[46]. The clinical and molecular differences of these syndromes will be one of the topics of this thesis.

After differentiation in the three axes of development, the hand is further formed by a self-induced outgrowth of the digits and controlled apoptosis in the interdigital spaces[47,48]. Both these processes are least well known. The self-induced outgrowth and condensation of the digits is controlled by the phalanx forming region (PFR), which shows strong activity of the BMP signaling pathway. Clinically, this can be observed by mutations in *IHH* and *ROR2*, which both cause different types of brachydactyly[49,50].

The exact mechanism of controlled apoptosis has fascinated scientists since the start of developmental biology and is one of the critical processes of hand development that is now also studied using the Turing mechanism[51,52]. For the purpose of this thesis, it is sufficient to appreciate that an intact AER (producing FGFs) is protective for interdigital apoptosis, whereas high doses of BMP induces apoptosis. Clinically this is relevant because mutations in FGF-receptors (e.g., Apert and Pfeiffer syndrome, both caused by *FGFR2* mutations) often present with syndactyly phenotypes due to failed apoptosis[53,54]. Other genes essential in this process, independently of which theorem will be proven correct, are *WNT* and SOX9.

The Oberg Manske and Tonkin classification.

Using the critique on the past classifications and the improved understanding of embryology, the Oberg Manske and Tonkin classification was developed. The OMT classification has three main goals[55]:

1. To provide a classification of congenital hand and upper limb anomalies which relates to our increased understanding of the etiology of anomalies and to relate this understanding to a determination as to which axis of development and differentiation is primarily involved, and whether the problem involves the whole of the upper limb or the hand plate primarily.
2. To allow documentation of all anomalies presenting in a single limb. For instance, if polydactyly, cleft hand, and syndactyly occur together, this is simply classified under "cleft hand complex." However, if a limb presents with apparently unassociated anomalies, such as syndactyly and clinodactyly, these are documented separately. Therefore, each limb may have multiple entries within the system.
3. To cross-reference anomalies with syndromes. If an anomaly is part of a syndrome, the specific anomaly is documented, as is the syndrome, and the two are cross-referenced. For instance, Holt-Oram syndrome is documented, and the limb anomaly - radial longitudinal deficiency (entire upper limb or hand plate alone) - is also documented.

Besides these three explicitly stated goals, the OMT classification also is designed to be used by multiple disciplines, contributing to a large variety of experience and interests among its users, as illustrated by fig 2.

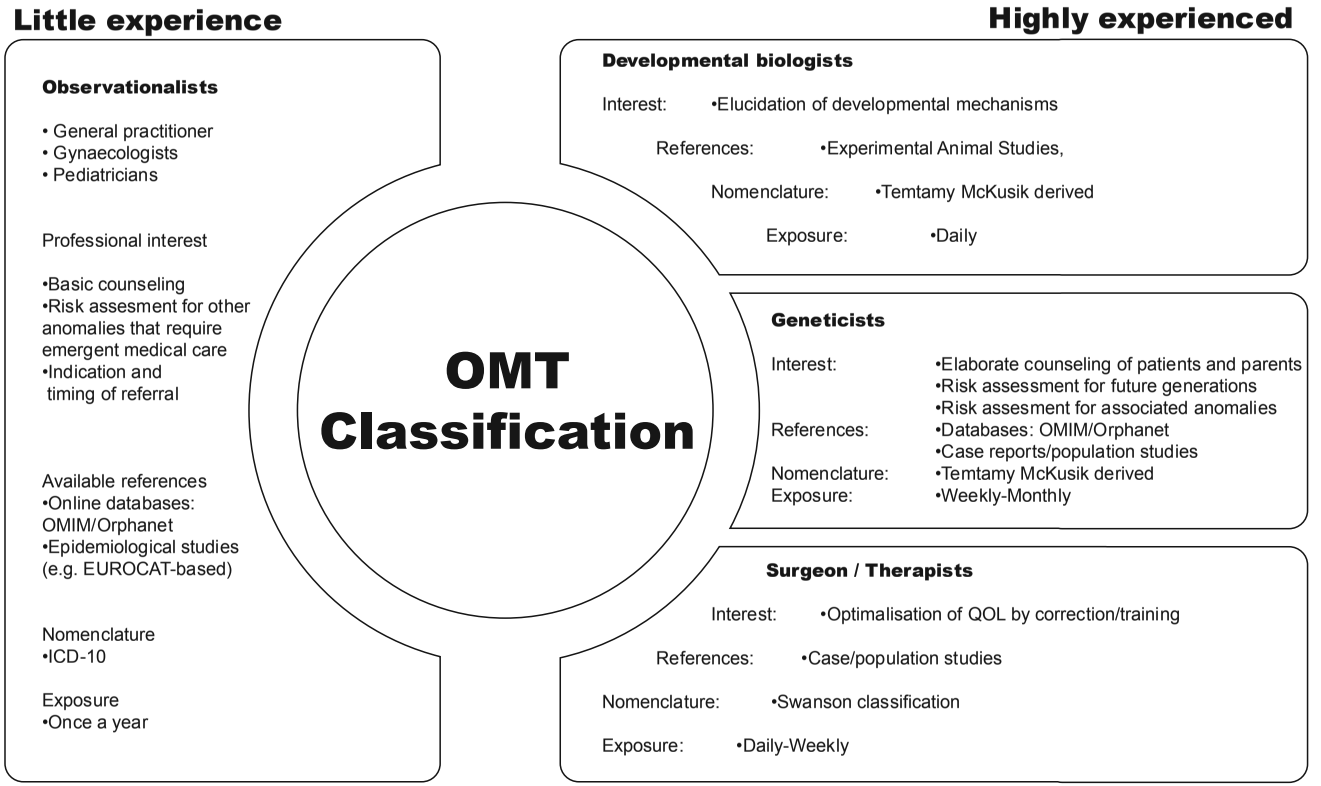


Figure 2. The OMT classification is designed for interdisciplinary use. This figure illustrates the aimed users, their exposure and experience, and the nomenclature used by training.

Structure of the OMT classification scheme

The OMT classification divides CULA into three groups using dysmorphological terminology. This leads to the following groups: *malformations, deformations,* and *dysplasia.* Syndromes are classified separately in a fourth group; however, it is aimed that any CULA is classified along with the observed syndrome. Dysmorphologies describe an additional term, *disruption*. However, as this process involves alteration of tissue already formed, Oberg et al. placed those conditions alongside those considered to be deformations.

The *malformations* category is reserved for conditions that are caused by aberrant limb formation. Before classifying the observed hand anomalies into the affected axis of development, first, a subdivision is made according to the extent of the developmental defect: entire limb or hand plate. Subsequently, the observed anomaly can be classified into any of the three axes of development: *proximal-distal, radial-ulnar (anteroposterior), dorsal-ventral.* Additionally, an unspecified axis group is present. This group is reserved for anomalies such as syndactyly, for which no specific axis can be determined. Furthermore, this group guarantees some flexibility in the classification.

The *deformations* category consists of conditions caused by deformation or disruption of normal limb development and is subdivided into three groups: constriction ring sequence, trigger digits, and not otherwise specified. The *dysplasia* category includes conditions associated with cellular atypia or tumor formation and is subdivided into hypertrophy and tumorous conditions, which both have subdivisions to specify the extent of hypertrophy or the nature of the tumorous condition. The syndromes category includes generalized syndromes that affect the upper limb

The consequence of this completely new method of classifying hand anomalies is illustrated in Table 2. Using the same example diagnoses from table 1, the consequences of this different approach are illustrated. For example, radial polydactyly and triphalangeal thumb are now considered disruptions of the radio-ulnar axis of development in the hand plate, whereas in the previous classification, these were two different entities. To put these two malformations together in one group not only makes more sense from a molecular point of view, but it is also what we observe in daily practice, e.g., in triphalangeal thumb synpolydactyly syndrome (OMIM:174500)[56].

Table 2. Simplified Oberg, Manske and Tonkin Classification

Classes			Example phenotypes / hand anomalies
Malformation	Upper limb	Proximal-distal	**Poland syndrome*** **Symbrachydactyly***
		Radial-ulnar	
		Dorso-ventral	
		Unspecified	
	Handplate	Proximal-distal	**Brachydactyly**
		Radial-ulnar	**Radial polydactyly*** **Triphalangeal thumb*** **Thumb deficiency***
		Dorso-ventral	
		Unspecified	**Syndactyly** **Central polydactyly**
Deformation			**Constriction ring syndrome**
Dysplasia			**Macrodactyly**

*hand anomalies that were not in classified in the same class in the IFSSH/Swanson classication

Classification strategy

The introduction of the OMT classification did not only comprehend a new classification scheme, but also the classification strategy has been altered. In the IFSSH classification, many were used to classify one key anomaly to describe the observed phenotype. In the OMT classification, a combination of diagnoses can allow the clinician to fully express the extent of the hand anomaly. This gives a better stratification of the hand anomaly and how it should be treated. This new classification strategy thus implies a switch from a "lump"-strategy to a "split" classification strategy. Or, going back to our wine-paralogue: rich, wood-aged Chardonnay wines can still be from the Languedoc, but you are now also able to assign these characteristics to a new-world Californian wine. In the hand, Radial polydactyly can now be a standalone phenotype, an expression of triphalangealism, or a part of a GLI3 polydactyly phenotype with postaxial polydactyly and syndactyly. Although confirmation studies of the OMT classification exist, the classification strategy was regularly limited to "one deformity per affected arm" [1,2,22].

The third aim of the OMT classification is also to cross-reference anomalies with syndromes. This approach contrasts with that of the IFSSH classification, which aimed to select only one of the classification groups. For a patient with hypoplastic thumbs in Holt-Oram syndrome, this would either be a failure of formation or registered under generalized abnormalities and syndromes. Classification of both the syndrome and the anomaly has important advantages. Keeping record of hand anomalies in

cases with a syndrome provides a better insight into disease presentation than birth registries or case reports of syndromes. Birth registries are often very a-specific and to gain insight into case-specific information requires manual status review. Case reports are prone to publication bias. Unfortunately, the first reports using the OMT classification do not fully use this ability of the classification.

Distal outcomes of a classification

The sole purpose of a classification system is not to classify; A classification system must serve a purpose in a clinic or in science. Current studies have tested the usability of the OMT classification in clinical practice. However, for science, little use has been illustrated to this point. One of the important scientific uses could be the recognition of combinations of (hand) anomalies with the same genetic substrate; homogenous phenotypes are not seldomly caused by a homogenous genetic cause: the combination of pre- and postaxial polydactyly with syndactyly can be pathognomic for diseases such as PPD type 4 / Greig syndrome[46]. Unfortunately, the current presentation of data using the OMT classification does not allow for these kinds of analyses. This thesis will illustrate that in-depth registration of all anomalies of individuals might reveal structural changing patterns of hand anomalies within one population.

The cross-reference of syndromes to hand anomalies is another aim of the classification to improve its usability in science. Because the OMT classification was designed with embryology in mind, the observed anomalies might tell us more about the affected signaling pathways. A simple example is triphalangealism: a simple cross-reference can teach us that this anomaly can be seen in isolated form when an enhancer of SHH is mutated and in Holt Oram syndrome. This knowledge can give us direction for future diagnostics and experimental studies, especially regarding the new techniques of genetic research such as Next Generation Sequencing producing enormous quantities of data that have to be filtered to detect the causative variant.

Next-Generation Sequencing (NGS)

Since the introduction of DNA sequencing, the field of genomics has evolved dramatically[57]. Sequencing technology was first successfully applied in 1951 by Sanger for the sequence of Insulin[58]. After proteins, the first RNA was successfully sequenced, followed by 12 bases of DNA in 1968[57]. Today, NGS allows us to sequence all 3 billion base pairs (and additional coverage to correct misreading) within hours. This evolution has influenced medical research and diagnostics. At first, genomic research was only available for large pedigree families that were suitable for linkage analysis. If a candidate gene or region was then identified, Sanger sequencing was applied to

determine the exact variation. Later, the direct evaluation of known disease-causing genes became available for diagnostics. With the current prices under $1000, one would expect this to be the new standard of care in diagnostics. However, NGS has presented new challenges in bioinformatics, ethics, and diagnostics. The critical problem is the excess of data obtained: NGS produces enormous data files; most of this data is entirely normal and therefore not of interest for diagnostics. The analysis of this data thus requires structural processing of the obtained reads, a comparison to reference genomes, annotation, and pedigree analysis.

Since the introduction of NGS, multiple efforts to produce reference cohorts have been made, including the GoNL, 1000 genomes, and the Wellderly project[59-61]. The reference cohorts aid in understanding which variants are possibly pathogenic and which are non-pathogenic variants based on, e.g., allele frequency in these healthy cohorts. Furthermore, the obtained data has allowed bioinformaticians to build tools that predict the pathogenicity of variants in genes, such as SIFT and PolyPhen-2, and even integrated scores such as CADD, which combine the prediction of multiple tools[62-64]. Together, these steps in the analysis and the tools provided by bioinformaticians form a pipeline for filtering NGS data and produce files with annotated variants observed in the patient of interest. Although these tools and cohorts help filter out or prioritize specific variants, these algorithms are still generic and non-disease specific; therefore, the filtered variants you might obtain can be disease-causing but related to a different disease that is not related to the diagnostic query, so-called incidental findings. This last concern often results in diagnostic panels that only provide information related to the disease of interest, therewith in-part ignoring the potential of genome-wide, unbiased diagnostics.

The use of sequencing in patients with CULA is still very sparse. Diagnostic panels have been evaluated before, with limited yield: e.g., Xiang et al. report an observed variant in 8/102 patients with radial polydactyly using GLI3, ZRS, preZRS, or SHH in their panel[65]. However, the pathogenicity of these mutations was not confirmed. Furniss et al. have screened a more heterogeneous group of CULA using a panel of 13 different genes and diagnosed 24/202 patients[66]. Other than these studies, few other population-based studies report the yield of genome-wide diagnostics, panel-based or using NGS, in patients with CULA. Likely, this is because of the limited group of patients with multiple congenital anomalies, the limited gain for patients, the risk for incidental findings, and the costs of NGS. However, scientifically the use of NGS in this population could still teach us substantially more about the embryology of the limb and thus improve the classification. The new OMT classification, in combination with related syndromes, could guide the way in CULA based analysis of NGS data, thus being another benefit of this classification over the Swanson classification and its concordant classification strategy. In this thesis, NGS will be used to provide unbiased genetic insight into the observed anomalies in our population.

Above and Beyond the Classification of Congenital Upper Limb Anomalies.

The newly introduced OMT classification has refueled the debate on the classification of CULA. Although validations for the classification scheme have been published, motivation and implementation for the new classification strategy is still lacking. The distal outcomes anticipated by using the new classification in cohort or genetic studies, are yet to be published. However, these would allow us to go above and beyond the classification and shift the balance in this discussion. Therefore, the aims of this thesis are:

To improve the understanding of the relation between CULA and underlying pathology, by:

- The cross-reference clinically observed hand anomalies to their underlying syndromes the OMT classification
- improving the availability of data on underlying syndromes of the CULA in the OMT classification

To evaluate the necessity of documenting all hand anomalies in patients with CULA by:

- reviewing the consequences of misclassification of a syndrome
- validating the clinical suggestion of increasing complexity of triphalangeal thumb phenotypes in TPT families
- developing clinical guidelines for the evaluation of children with medial polydactyly based on our local cohort and our in-house developed methodology.

To illustrate the value of in-depth phenotypic description for molecular diagnostics, by:

- studying the patterns of polydactyly in patients with GLI3 mediated polydactyly syndromes
- studying the molecular origin of complex TPT phenotypes in a large pedigree family with no ZRS mutations.
- developing clinical guidelines for the evaluation of children with RLD based on our local cohort and by studying the molecular origin of RLD using NGS

Globally, this thesis describes the path from the observation of a limb anomaly, its classification, the clinical or diagnostic consequences, and finally the molecular analysis. This connection between clinical observations, the clinical consequences and the underlying pathology is necessary to go above and beyond the act of classification itself and is explored in the three parts of this thesis.

Part 1 is on the classification of limb anomalies and is comprised of 3 chapters. **Chapter one** evaluates the new combined diagnosis registration theory of the new OMT classification. **Chapter two** deals with the cross-reference of syndromes to the OMT classification. In **chapter three**, Poland syndrome is used to illustrate the diagnostic challenges when we "under-classify" observed anomalies as the same syndrome. **Part 2** of the thesis underlines the importance of detailed classification and the cross-reference with syndromes based on two common clinical phenotypes in our practice and is comprised of 2 chapters. **Chapter 4** deals with a new observation of the increased complexity of the malformations in our triphalangeal thumb cohort in Rotterdam. **Chapter 5** uses the methodology presented in chapter two to provide a broad differential diagnosis for patients with medial polydactyly of the foot, a commonly encountered malformation in patients with Greig syndrome. **Part 3** of this thesis uses the detailed phenotyping established in the previous chapters of this thesis and relates this knowledge to the patients' genotype. In **chapter 6**, we learn that the detailed phenotype observed in Greig syndrome relates to the position and effect of the GLI3 mutation. **Chapter 7** revisits the triphalangeal thumb cohort and finds a new regulatory element that also affects SHH regulation similar to the ZRS. Finally, in **chapter 8**, we use Next Generation Sequencing to determine its yield in patients with radial longitudinal deficiency.

PART 1

Classification of CULA using the OMT classification

2

Documenting Combined Congenital Upper Limb Anomalies Using the Oberg, Manske, and Tonkin Classification: Implications for Epidemiological Research and Outcome Comparisons

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ABSTRACT

Purpose: Congenital upper limb anomalies (CULAs) exhibit a wide spectrum of phenotypic manifestations. To help the clinician evaluating this variety of CULAs, the Oberg, Manske, and Tonkin (OMT) classification was recently introduced. The OMT classification allows for documentation of combined hand anomalies. However, subsequent epidemiological and validation studies using the OMT scheme commonly registered only the main anomaly per arm. This study illustrates both the deficits of single diagnosis documentation as well as the merits of registering every anomaly for epidemiological research, outcome comparison, and overall applicability of the classification.

METHODS: We retrospectively reviewed patients visiting the Erasmus MC - Sophia Children's Hospital between 2012 and 2014. All congenital anomalies of both limbs were classified according to the OMT scheme. The frequency of combined diagnoses as well as recurrent combinations were analyzed. The relation to the co-registered syndromes was studied.

RESULTS: We included 746 patients, 79.5% of whom could be documented with a single OMT diagnosis. In 20.5%, a combination of OMT diagnoses was documented. We documented 149 different combinations: 102 were documented once, 47 were documented repeatedly (n 1⁄4 196); for example, in patients with Greig syndrome. The prevalence of this syndrome was significantly higher in patients with a combination of radial polydactyly, ulnar polydactyly, and/or syndactyly (2.9% vs 33.3% and 60% in patients with 1 vs 2 and 3 diagnoses).

CONCLUSION: Documentation of combined OMT diagnoses is required in a fifth of the patients. Not doing so will cause loss of phenotypic information and can hamper outcome comparison and epidemiological research. Documentation of combined OMT diagnoses can help to identify subgroups within a population, for example, patients with an underlying syndrome. Last, combined documentation of diagnoses improves flexibility of the classification and thereby better allows universal application.

Clinical relevance: Consensus on the application of the OMT classification is critical to achieving the universal adoption of the system by hand surgeons and other medical professionals.

INTRODUCTION

Congenital upper limb anomalies (CULAs) exhibit a wide spectrum of phenotypic manifestations[67]. This variability can be observed in both the severity, as well as the embryological nature of the anomaly[20]. To facilitate outcome comparison and epidemiological research, multiple classification and registration systems have been developed in the past, such as the Swanson classification[7,9,16,17,20,68]. However, as the understanding of both embryology increased, classification issues arose[7,18,19]. To resolve these issues, the Oberg, Manske and Tonkin (OMT) classification was developed[7,10,20,21]. The OMT-classification aims to: 1) provide a classification which fits the current concepts of etiology, 2) allow combined documentation of all anomalies present in a single limb, 3) cross-reference anomalies with syndromes[5].

The introduction of the OMT-classification was succeeded by multiple validation studies[1,2,21,22]. Most of these studies aimed to test usability or reliability of the classification. However, these studies are inconsistent regarding combined documentation of anomalies: Tonkin and Oberg used multiple OMT-diagnoses within one limb if necessary[21], whereas both Goldfarb et al. and Ekblom et al. only registered two diagnoses in bilateral asymmetrically affected patients[1,2]. Moreover, Ekblom et al. only classified the second anomaly when it was classified in a different OMT-category[2].

Documentation of combined anomalies allows the clinician to register all clinical aspects of compound phenotypes that may influence decision making with respect to both treatment options and diagnostics, such as an echocardiogram. Not doing so could result in the loss of clinically relevant phenotypic information. For example, in patients with a triphalangeal thumb, the presence of synpolydactyly is a reason for a 2-stage operation, which is likely to influence surgical outcomes. Alternatively, the presence of a triphalangeal thumb in co-occurrence with radial longitudinal deficiency is highly suspicious for Holt-Oram syndrome[69]. Depending on the classification strategy used in the previous studies, both examples would be classified as either 1 diagnosis (Triphalangeal Thumb or Synpolydactyly and Triphalangeal Thumb or Radial Longitudinal Deficiency, respectively) or 2 diagnoses (Triphalangeal Thumb with Synpolydactyly and Radial Longitudinal Deficiency with Triphalangeal Thumb, respectively) in a single limb.

Documentation of combined OMT diagnoses has never been evaluated. However, studying combined diagnoses could help to improve the OMT classification scheme and the classification strategy in multiple ways. First, quantification of the number of combined diagnoses helps to evaluate its necessity. Second, the presence of recurring combinations of diagnoses could indicate a missing OMT diagnosis or sub diagnosis, like Poland syndrome as a sub-diagnosis for symbrachydactyly.

Last, the cross-referencing of the observed combined diagnoses to syndromes can provide indicators for an underlying etiology, as it does in the Holt-Oram example. The aim of our study was to evaluate the use of combined OMT diagnoses. Secondarily, we aimed to identify hand anomalies that could not be classified in the current OMT classification scheme.

METHODS

Sample

We retrospectively reviewed all patients visiting the Erasmus MC Team for congenital hand and upper limb differences in the Erasmus MC - Sophia Children's Hospital between January 2012 and December 2014. Patients visiting the outpatient clinic for a first consultation and patients visiting as part of follow-up were eligible for inclusion. Patients with acquired anomalies, traumas, or isolated anomalies of the feet were excluded; patients suffering from a systemic condition also affecting the upper extremity (e.g., Hereditary Motor and Sensory Neuropathy) were excluded as well. Patient characteristics, family his- tory, radiographs, clinical photographs, involvement of specialists other than hand surgeons, associated anomalies, and genetic work-up were obtained to allow for proper classification.

Classification strategy

At first consultation, a preliminary diagnosis for each patient was documented by 1 of the senior authors (S.E.R.H. and C.A.v.N.). This diagnosis was retrieved from medical records by M.B. and P.R.Z. and confirmed by cross-referencing with clinical and radiographic photos and the follow-up by the senior authors. Based on this information, patients were classified according to the modified OMT -classification as proposed by Tonkin et al in 2017[23]. All congenital anomalies of both limbs were classified according to the OMT scheme individually. In compound phenotypes, every anomaly present was classified, unless an overarching term was available, such as Synpolydactyly. In bilateral patients, both hands were documented separately. However, OMT diagnoses present in both hands were counted as 1 diagnosis. Therefore, the number of diagnoses obtained for a bilateral case was the number of unique OMT diagnoses in that individual. This classification strategy is in line with the advice from Tonkin regarding using the OMT scheme[23]. Anomalies, which did not evidently fit a certain diagnosis, were classified as an *otherwise not specified anomaly* as introduced by Goldfarb et al.[1]. The syndrome diagnosis was obtained from both clinical as well as genetic records. If the records and/or the molecular diagnosis were conflicting, the genetic diagnosis was regarded the correct syndrome diagnosis.

Combined diagnoses use

The frequency of combined diagnoses was defined as the documentation of more than 1 OMT diagnosis per patient. For the individual patients, there was no maximum number of OMT diagnoses to document their compound phenotype.

However, we analyzed the occurrence of any 2 OMT diagnoses combined in a patient, regardless of additional OMT diagnoses that might have been documented. Consequently, multiple combinations can occur in 1 patient. For example, in a patient with (1) Radial Polydactyly, (2) Ulnar Polydactyly, and (3) Syndactyly, 3 combinations can be made: 1 with 2, 1 with 3, and 2 with 3.

Recurring combinations were defined as any combination of OMT diagnoses observed in more than 1 patient. Recurring combinations were analyzed for all OMT diagnoses with a significantly higher prevalence of combined diagnosis usage. Furthermore, the application of combined diagnoses documentation was explored among patients with a co-registered syndrome and the effect of the different classification strategies was studied.

Statistical analysis

For statistical testing, a chi-square or Fisher exact test for group proportion differences was used. Combined diagnosis usage was considered a dichotomous variable. The structure of the OMT-classification was respected; thus, the prevalence of combined diagnosis usage in Malformations was compared with that in Deformations and Dysplasia, and so on. Subsequently, combined diagnoses in Radial Polydactyly were compared with that of other radial-ulnar malformations of the handplate. The threshold for significance was .05.

Ethics

Approval for this study was obtained from the local medical ethics committee (MEC-2015-012).

RESULTS

From January 2012 till December 2014, a total of 736 patients were eligible for inclusion, of which 375 were unilaterally affected (Right/Left: 180/195) and 361 patients were bilaterally affected. In these patients 952 OMT diagnoses were registered. In 227 cases, a syndrome was co-registered. The full list of anomalies is presented in Appendix A.

In 79.5% of the patients, a single OMT diagnosis could be used. In the remaining 20.5% patients, a compound phenotype was observed that required documentation of more than 1 OMT diagnosis. In patients with multiple diagnoses, 149 different combinations of OMT diagnoses were documented: 102 were documented once, 47 were documented recurrently. The 47 combinations that were documented recurrently, accounted for 65.8% of combined OMT diagnoses documentation (n=196). All combinations of OMT diagnoses encompassed at least 1 malformation.

Table 1 shows the diagnoses that were most frequently used in a combined diagnosis. Ventral dimelia, Clinodactyly, and Synostosis/Symphalangism showed no statistically significant difference in combined diagnoses usage to other diagnoses in their OMT class. However, owing to the (near) total contribution of those specific diagnoses to the total of observed diagnoses in that specific class, they were included for further analysis.

The most frequently observed combination of OMT diagnoses was Triphalangeal Thumb and Radial Polydactyly (23 out of 28 patients with Triphalangeal Thumb). Ulnar Longitudinal Deficiency and Radial Longitudinal Deficiency were both commonly documented combined with their related anomalies limited to the handplate: Ulnar Deficiency of the Handplate (7.7%) and Radial Deficiency of the Handplate (17%). Ulnar Deficiency of the Handplate and Ulnar Longitudinal Deficiency were both repeatedly combined with Syndactyly (38.7% and 66.7% of patients). Furthermore, Syndactyly was also combined with numerous different OMT diagnoses, most frequently with Ulnar Polydactyly and Radial Polydactyly (Table 2).

The use of combined OMT diagnoses was significantly higher in patients with a diagnosed syndrome (28.6% vs 17.6%; $P < .05$). Thirteen syndromes were registered more than once and, on average, required more than 1 OMT diagnosis (Table 3). Greig cephalopolysyndactyly syndrome was the most frequently observed syndrome. Patients with this syndrome typically presented with Radial Polydactyly, Ulnar Polydactyly, and Syndactyly. The prevalence of Greig syndrome was significantly higher in patients with 2 or more typical hand anomalies (Table 4; 2.9% vs 33.3% and 60% in patients with 1 vs 2, or 3 OMT diagnoses; $P < .05$).

Deficits of the OMT classification

We documented 3 patients in the Not Otherwise Specified Group of the OMT classification. Their clinical diagnoses were Congenital Compartment Syndrome, Lymphatic Malformation, and Central Polydactyly.

The diagnosis Aberrant Shoulder Muscles was registered 12 times, 10 of which clinically were hypoplastic pectoralis muscles (with a hand anomaly other than Symbrachydactyly).

Table 1. Combined diagnosis use per OMT class

			Number of diagnoses	Patients with 1 diagnosis	Patients with a combined diagnosis	
Overall			**952**	**579**	**155**	
1. Malformation			849	491	155	***
2. Deformation			68	62	6	
3. Dysplasia			34	28	5	
1A	**Malformation - Entire limb**		178	**109**	**48**	
	1	Proximal-distal axis	33	28	5	
	2	Radio-ulnar axis	98	50	41	***
		i Radial longitudinal deficiency	53	18	35	*
		ii Ulnar longitudinal deficiency	31	21	10	**
		vi Humero radial synostosis	4	0	4	*
	3	Dorso-ventral axis	2	2	0	
	4	Unspecified axis	45	29	16	
1B	**Malformation - Handplate**		671	**385**	**142**	
	1.	Proximal-distal axis	82	61	20	
	2.	Radio-ulnar axis	283	158	82	**
		ii Ulnar deficiency of the hand	*14*	2	12	
		v Triphalangeal thumb	*28*	3	25	
	3.	Dorso-ventral axis	8	0	8	***
		ii Ventral dimelia - incl. hypoplastic nail	8	0	8	
	4.	Unspecified axis	298	167	100	
		i. Soft tissue	*183*	102	71	***
		(a) Syndactyly	86	*35*	*51*	***
		ii. Skeletal	48	19	24	***
		(a) Clinodactyly	30	15	15	
		(c) Synostosis/ Symphalangism	13	3	10	
		iii. Complex	*67*	45	20	

Table 1. Combined diagnoses use for all classes of the OMT classification scheme. To improve comprehensibility, all OMT-classes and subclasses are depicted, however only the diagnoses with a significantly higher prevalence of combined diagnoses use are displayed. (* indicates a p-value of <0.05, ** p <0.01, *** p<0.001)

Table 2.Observed recurrent combinations of OMT diagnoses

Primary diagnosis	***was used in combination with***	***n***
Radial longitudinal deficiency (n=53)	Radial deficiency of the hand (contra-lateral)	6
	Pectoral hypoplasia	5
	Ulnar longitudinal deficiency	4
	Complex abnormalities of the cervical spine	3
	Syndactyly	3
	Radial polydactyly	2
	Synostosis/Symphalangism	2
Ulnar Longitudinal Deficiency (n=31)	Syndactyly (Complex or Simple)	12
	Radial Longitudinal deficiency	4
	Ulnar Deficiency of the Hand (Contralateral)	4
	Humeroradial Synostosis	3
	Radial Deficiency of the Hand	2
	Synostosis/Symphalangism	2
Humero-radial synostosis (n=4)	Syndactyly	2
Ulnar Deficiency of the Hand (n=14)	Syndactyly (Complex or Simple)	8
	Synostosis/Symphalangism	2
	Radial deficiency of the hand	2
Triphalangeal Thumb (n=28)	Radial Polydactyly	23
	Syndactyly (Complex or Simple)	6
	Ulnar Polydactyly	4
Ventral dimelia - Hypoplastic Nail (n=8)	Brachydactyly	3
	Syndactyly	2
Syndactyly (n=86)	Radial polydactyly	14
	Ulnar Polydactyly	13
	Complex Syndactyly	4
	Brachydactyly	4
	Windblown Hand	3
	Radio-ulnar synostosis	2
	Radial deficiency of the hand	2

Primary diagnosis	***was used in combination with***	***n***
	Clinodactyly	2
	Synostosis/Symphalangism	2
	Brachydactyly	2
Clinodactyly (n=30)	Brachydactyly	4
	Camptodactyly	3
	Radial deficiency of the hand	2
	Radial Polydactyly	2

Table 2 displays the most commonly combined OMT diagnoses obtained from Table 1. *Structural combinations are only noted once, thus *symphalagism/ synostosis* is not included as a primary diagnosis.

Table 3. Observed hand anomalies in syndromic cases with combined diagnoses

	Proteus	Ulnar-mammary	MASA	Bardet-Biedl	Craniofrontonasal dysplasia	Greig cephalopolysyndactyly	Holt-Oram	Nager	EEC	Freeman Sheldon	Klippel Feil	Distal arthrogryposis	VACTERL association
	35.	43.		4.		17.	19.	25.	12.			10.	44.
Patients (n)	**2**	**2**	**2**	**3**	**3**	**17**	**5**	**2**	**2**	**2**	**2**	**24**	**14**
Diagnosis (n)	**6**	**6**	**5**	**7**	**7**	**34**	**10**	**4**	**3**	**3**	**3**	**28**	**16**
Radial longitudinal deficiency							5	1			1		7
Ulnar longitudinal deficiency		1						1					
Complex anomalies of the cervical spine	1										1		
Pectoral hypoplasia							2						
Abberant shoulder muscles					1								
Brachydactyly		1		1								1	
Radial deficiency of the hand							1	1				1	6
Ulnar deficiency of the hand		1											
Radial polydactyly					2	14	1				1		3
Triphalangeal thumb					1		1	1					
Ulnar polydactyly		1		2	1	9							
Hypoplastic nail					1	1							
Syndactyly	1	1	1	1	1	8						1	
Camptodactyly										1			
Thumb in palm deformity			2									1	
Arthrogryposis/ Windblown hand			2							2		24	
Synostosis / symphalagism				1									
Syndactyly with synostosis of phalanges						2							

	Proteus 35.	Ulnar-mammary 43.	MASA	Bardet-Biedl 4.	Craniofrontonasal dysplasia	Greig cephalopolysyndactyly 17.	Holt-Oram 19.	Nager 25.	EEC 12.	Freeman Sheldon	Klippel Feil	Distal arthrogryposis 10.	VACTERL association 44.
Patients (n)	**2**	**2**	**2**	**3**	**3**	**17**	**5**	**2**	**2**	**2**	**2**	**24**	**14**
Diagnosis (n)	**6**	**6**	**5**	**7**	**7**	**34**	**10**	**4**	**3**	**3**	**3**	**28**	**16**
Cleft		1							2				
Synpolydactyly				1					1				
Hemi-hypertrophy	1												
Macrodactyly	1												
Vascular malformation	1												
Osteochondroma	1												

Table 3 Shows the distribution of hand anomalies among syndromes. Only syndromes observed more than once with more than 1 diagnosis per patient on average were included in this table.

Table 4. Number of typical hand anomalies for Greig syndrome and the prevalence of Greig syndrome

Number of typical Greig hand anomalies	1	2	3
Number of patient meeting criteria	212	24	5
Number of Greig patients	6	8	3
Prevalence of Greig	2.88%	33.33%	60.00%
			***

Table 4. Number of typical hand anomalies for Greig syndrome and the prevalence of Greig syndrome. The hand features selected for this comparison were: *Radial polydactyly*, *Ulnar polydactyly* and *Syndactyly*. There is a statistically higher prevalence of Greig syndrome among patients with 2 or more of these hand anomalies. (*** indicates a p-value of <0.001)

DISCUSSION

In this study, we evaluated documentation of combined OMT diagnoses. In 20.5%, of the included patients, phenotypic information would have been lost if combined diagnoses had not been used. Such substantial loss of phenotypic information could hamper epidemiological and outcomes research. For example, patients with a Triphalangeal Thumb with or without Radial Polydactyly may require a different surgical approach and subsequently have different surgical outcomes. In addition, repeated observation of combinations of diagnoses might also indicate the presence of a subgroup with a common etiology, as illustrated by the increased prevalence of Greig syndrome among patients with a combination of Radial Polydactyly, Ulnar Polydactyly, and Syndactyly. From a more pragmatic point of view, combined diagnosis documentation also might lessen the need for revisions of the OMT classification; replacing the recurrent combinations in our study for fixed diagnoses, would imply introducing 47 new entities to the classification. Over time, this approach could lead to unnecessary revisions of the classification. Therefore, the use of combinations of OMT diagnoses has merit and should be applied to communicate and detect the nuances in disease presentation that might have possible implications for surgery or research and to prevent unnecessary revisions to the classification.

As expected, the number of diagnoses documented in our population was considerably higher than that found in other studies that classified a single diagnosis per arm[1,2], but lower than that reported by Tonkin et al[21]. Tonkin et al. documented and reported the number of anomalies per limb, thereby not correcting for symmetrically bilateral anomalies, which resulted in 177 diagnoses in 101 patients. In all other studies, including ours, a symmetrical bilateral anomaly was counted as 1 diagnosis in 1 patient. The observed frequency of combined diagnoses might raise concerns regarding over-stratification; combining OMT diagnoses could create too many subgroups, restricting the number of patients available for e.g. outcome comparison. Theoretically, the 60 OMT diagnoses could produce over 1770 combinations. In our study, we observed only 149 of these 1770 combinations. Furthermore, combinations that were recurrently observed accounted for 65.8% of all combined diagnoses. It is likely that the number of sub-groups created by combining OMT diagnoses is limited and the benefits of documenting combined OMT diagnoses thus outweigh the possible stratification issues.

Population specific classification difficulties may arise each time the OMT classification is applied, the flexibility provided by documentation of combined diagnoses prevents unnecessary adaptations of the classification due to these difficulties. In our study, there were 47 recurrent combinations of OMT diagnoses, the most frequently applied combination being Triphalangeal Thumb with Radial

Polydactyly. These anomalies are highly prevalent due to a genetic isolate in the southern Netherlands[70]. Subsequently, we could advocate adding a fixed term for the complex Triphalangeal Thumb phenotypes to resolve our classification issue. However, this example does not stand by itself; many other adaptations could be advocated, which would introduce unnecessary revisions of the classification. For example, Ulnar Longitudinal Deficiency and Ulnar Deficiency of the Handplate were documented combined with Syndactyly in 38.7% and 66.7% of the patients. Subsequently, we could also endorse adding sub-diagnoses to these diagnoses, like the sub-diagnoses which are already available for Radial Longitudinal Deficiency (e.g. Absent thumb). Arguably, both examples indicate the presence of different sub-groups within our population; however, we were able to document the observed anomalies using a combination of OMT diagnoses. Therefore, we prefer to use documenting combined OMT diagnoses over revision of the OMT classification scheme to prevent adaptation based on local observations.

As one might expect, combined OMT diagnoses were more frequently observed in syndromic patients. Studying these hand anomalies in relation to the underlying syndrome might enable the development of clinical guidelines for patient referral. To achieve this, we need standardized reporting on our syndromic patients. Although cross-referencing of anomalies to the underlying syndromes is one of the main aims of the OMT classification, currently most authors only report the observed prevalence of syndromes, not the observed hand anomalies per syndrome. Some might not agree that the development of such guidelines is the domain of the surgeon. However, no other medical specialist is confronted with this unique case mix of both syndromic and non-syndromic CULA patients, thus the surgical population is most suitable for this differentiation. Alternatively, Lowry et al. suggest these cases could be studied using ICD-10 based birth registries[71]. In line with Tonkin's response to Lowry et al., our study shows that accurate classification is key in epidemiological research and the lack of specificity of the ICD-10 classification is unacceptable[72]. For example, in an ICD-10 based study in the Northern Netherlands, the birth prevalence of triphalangeal thumbs was not reported because of the lack of a suitable ICD-10 term. Manual review of the syndromic cases in this study revealed that triphalangeal thumbs were present in the underlying population[3]. Consequently, due to the lack of overall birth prevalence of triphalangeal thumbs, no measure of risk for the associated syndromes can be calculated.

There were several entities that are hard to classify. To resolve these issues Central Polydactyly, meaning cases without syndactyly, should be added to complex malformations of the hand plate. Embryologists might argue Central Polydactyly can be classified as a Cleft Hand because of their suggested shared pathophysiology. However, for the surgical application of the classification we feel

it is justified to differentiate between the two entities. Furthermore, Lymphatic Tumors should be added to the OMT classification. In concordance with the International Society for the Study of Vascular Anomalies, we propose Lymphatic Tumors should be added to the vascular tumors class[73].

Last, to prevent classification errors in patients with hypoplasia of the pectoralis muscle, we suggest adding Pectoral Aplasia/Hypoplasia as a separate entity to Shoulder Malformations. Goldfarb et al. introduced Poland syndrome as a sub-diagnosis of Symbrachydactyly[1]. However, the included patients presented with different malformations, such as Radial Longitudinal Deficiency and Ulnar Longitudinal Deficiency. These combinations can be observed in other syndromes as well, such as Ulnar Mammary Syndrome. Therefore, using Poland syndrome for the classification of these patients would be incorrect. On the other hand, only classifying Abnormal Shoulder Muscles would be too non-specific[74].

Documentation of combined OMT diagnoses is an essential feature of the OMT classification. Single diagnosis classification strategies lead to a loss of phenotypic information in a substantial number of patients and will hamper outcome comparisons and research, especially epidemiological studies. Furthermore, reporting on the documented combinations can provide valuable insight into syndromic patients. Thus, future registries and studies should report single and combined OMT diagnoses.

Appendix A: Full OMT-classification scheme including the number of affected cases, laterality and mean number of diagnosis per class and diagnose. CI = confidence interval.

OMT classification		Right	Left	Bilateral	Mean	upper limit 95% CI	lower limit 95% CI
Overall		**218**	**230**	**504**	**1.3**	**1.35**	**1.25**
1. Malformations		187	200	462	1.34	1.39	1.28
1A	**Failure of axis formation/differentiation – entire upper limb**	45	47	86	1.5	1.64	1.36
	Proximal-distal axis	13	19	1	1.21	1.42	1
	Brachymelia	2	0	0	2.5	21.56	-16.56
	Symbrachydactyly	6	9	0	1.07	1.21	0.92
	a) Poland syndrome	6	8	0	1.07	1.23	0.92
	b) Whole limb excluding Poland syndrome	0	1	0	1	1	1
	Transverse deficiency	5	9	1	1.13	0.94	1.33
	Intersegmental deficiency	0	1	0	1	1	1
	Whole limb duplication/triplication	0	0	0	0	0	0
	Not otherwise specified	0	0	0	0	0	0
1A2	**Radioulnar axis**	27	20	51	1.77	1.98	1.55
	Radial longitudinal deficiency	11	10	32	1.57	1.82	1.31
	Ulnar longitudinal deficiency	13	8	10	2.13	2.49	1.77
	Ulnar dimelia	0	0	0	0	0	0
	Radioulnar synostosis	2	1	4	2	3.07	0.93
	Congenital dislocation of the radial head	0	0	1	3.5	3.5	3.5
	Humeroradial synostosis - Elbow ankylosis	1	1	2	3.25	5.25	1.25
	Madelung deformity	0	0	2	1	1	1
	Not otherwise specified	0	0	0	0	0	0

OMT classification		Right	Left	Bilateral	Mean	upper limit 95% CI	lower limit 95% CI
Overall		**218**	**230**	**504**	**1.3**	**1.35**	**1.25**
1A3	**Dorsal-ventral axis**	0	0	2	1	1	1
	Ventral dimelia	0	0	0	0	0	0
	a) Fuhrmann/Al-Awadi/Raas-Rothschild syndromes	0	0	0	0	0	0
	b) Nail-patella syndrome	0	0	0	0	0	0
	Aberrant extensor/flexor muscles	0	0	2	1	1	1
	Not otherwise specified	0	0	0	0	0	0
1A4	**Unspecified axis**	5	8	32	1.6	1.89	1.3
	Shoulder	5	7	8	2.33	3.07	1.6
	a) Undescended shoulder (Sprengel deformity)	0	0	1	1	1	1
	b) Pectoral hypoplasia/aplasia	3	4	3	2.5	3.34	1.66
	c) Abnormal shoulder muscles	0	0	2	3	15.7	-9.7
	d) Not otherwise specified abnormalities of cervical spine and shoulder	2	3	2	2.71	3.99	1.44
	Arthrogryposis	0	1	24	1.08	1.19	0.97
	Not otherwise specified	0	0	0	0	0	0
1B	**Failure of axis formation/differentiation – hand plate**	142	153	376	1.38	1.44	1.32
1B1	**Proximal-distal axis**	23	36	23	1.36	1.52	1.19
	Brachydactyly	8	5	20	1.82	2.17	1.47
	Symbrachydactyly	14	20	2	1.03	1.08	0.97
	Transverse deficiency	1	11	1	1.23	1.5	0.97
	Not otherwise specified	0	0	0	0	0	0
1B2	**Radioulnar axis**	76	75	132	1.51	1.62	1.4

OMT classification		Right	Left	Bilateral	Mean	upper limit 95% CI	lower limit 95% CI
Overall		**218**	**230**	**504**	**1.3**	**1.35**	**1.25**
	Radial deficiency	11	11	28	1.66	1.96	1.36
	Ulnar deficiency	5	4	5	2.86	3.67	2.05
	Radial polydactyly	49	39	36	1.53	1.67	1.39
	Triphalangeal thumb	8	4	16	2.36	2.68	2.04
	Ulnar dimelia (mirror hand)	0	0	0	0	0	0
	Ulnar polydactyly	3	17	47	1.55	1.77	1.33
	Not otherwise specified	0	0	0	0	0	0
1B3	**Dorsal-ventral axis**	1	1	6	2.63	3.51	1.74
	Dorsal dimelia	1	0	0	3.5	3.5	3.5
	Ventral dimelia	0	0	0	0	0	0
	Nail aberrations	0	1	6	2.29	2.74	1.83
	Not otherwise specified	0	0	0	0	0	0
1B4	**Unspecified axis**	42	41	215	1.57	1.68	1.46
	Soft tissue	23	30	130	1.64	1.78	1.5
	Syndactyly	13	20	53	1.97	2.18	1.75
	Camptodactyly	8	6	31	1.36	1.62	1.09
	Deviated finger without skeletal	1	0	2	1	1	1
	Thumb in palm deformity	1	2	12	1.67	2.16	1.17
	Distal arthrogryposis	0	2	32	1.4	1.61	1.19
	Not otherwise specified	0	0	0			
	Skeletal deficiency	11	5	32	2.02	2.4	1.65
	Clinodactyly	6	2	22	1.87	2.31	1.42

OMT classification			Right	Left	Bilateral	Mean	upper limit 95% CI	lower limit 95% CI
Overall			**218**	**230**	**504**	**1.3**	**1.35**	**1.25**
		Kirner deformity	0	1	0	1	1	1
		Synostosis/Symphalagism	5	2	10	2.75	5.47	0.03
		Not otherwise specified	0	0	0	0	0	0
	Complex		8	6	53	1.46	1.68	1.25
		Complex syndactyly	2	1	10	2	2.43	1.57
		Cleft hand	1	2	12	1.47	2.19	0.75
		Synpolydactyly	3	1	9	1.85	2.33	1.36
		Central polydactyly	0	1	0	2	2	2
		Complicated syndactyly	2	1	22	1	1	1
		Not otherwise specified	0	0	0	0	0	0
2. Deformations			23	16	30	1.1	1.19	1.02
	Constriction ring sequence		7	6	24	1.14	1.27	1
	Trigger digits		16	9	6	1.13	0.97	1.29
	Congenital compartment syndrome		0	1	0	0	0	0
3.Dysplasia			8	14	12	1.3	1.62	0.99
3A	**Hypertrophy**		2	9	3	1.38	1.91	0.86
	Whole limb		0	2	1	2.33	6.13	-1.46
		Hypertrophy	0	1	1	3	15.71	-9.71
		Aberrant flexor/extensor/intrinsic muscles	0	1	0	1	1	1
	Partial limb		2	7	2	1.18	1.45	0.91
		Macrodactyly	1	6	2	1.22	1.56	0.88

OMT classification			Right	Left	Bilateral	Mean	upper limit 95% CI	lower limit 95% CI
Overall			**218**	**230**	**504**	**1.3**	**1.35**	**1.25**
		Aberrant intrinsic muscles of hand	1	1	0	1	1	1
3B	**Tumorous conditions**		6	5	9	1.32	1.71	0.92
3B1	**Vascular**		2	3	1	1.5	2.79	0.21
		Hemangioma	0	0		0	0	0
		Malformation	2	2	1	1.5	2.79	0.21
		Lymfatic malformation	0	1	0	1	1	1
		Other	0	0	0	0	0	0
3B2	**Neurological**		1	0	0	1	1	1
		Neurofibromatosis	1	0	0	1	1	1
		Other	0	0	0	0	0	0
3B3	**Connective tissue**		1	1	1	2.33	8.07	-3.4
		Juvenile aponeurotic fibroma	0	0	0	0	0	0
		Infantile digital fibroma	1	1	1	2.33	8.07	-3.4
		Other	0	0	0	0	0	0
3B4	**Skeletal**		2	1	7	1.4	2.09	0.71
		Osteochondromatosis	1	0	5	1.5	2.79	0.21
		Enchondromatosis	1	0	1	0	0	0
		Fibrous dysplasia	0	1	0	1	1	1
		Epiphyseal abnormalities	0	0	1	2	2	2
		Other	0	0	0	0	0	0

3

Identification of Associated Genes and Diseases in Patients With Congenital Upper-Limb Anomalies: A Novel Application of the OMT Classification

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ABSTRACT

PURPOSE: Congenital upper-limb anomalies (CULA) can present as a part of a syndrome or association. There is a wide spectrum of CULA, each of which might be related to different diseases. The structure provided by the Oberg, Manske, and Tonkin (OMT) classification could aid in differential diagnosis formulation in patients with CULA. The aims of this study were to review the Human Phenotype Ontology (HPO) project database for diseases and causative genes related to the CULA described in the OMT classification and to develop a methodology for differential diagnosis formulation based on the observed congenital anomalies, CulaPhen.

METHODS: We reviewed the HPO database for all diseases, including causative genes related to CULA. All CULA were classified according to the OMT classification; associated non-hand phenotypes were classified into 12 anatomical groups. We analyzed the contribution of each anatomical group to a given disease and developed a tool for differential diagnosis formulation based on these contributions. We compared our results with cases from the literature and with a current HPO tool, Phenomizer.

RESULTS: In total, 514 hand phenotypes were obtained, 384 of which could be classified in the OMT classification. A total of 1,403 diseases could be related to those CULA. A comparison with 10 recently published cases with CULA revealed that the presented phenotype matched the descriptions in our dataset. The differential diagnosis produced using our methodology was more accurate than Phenomizer in 4 of 5 examples.

CONCLUSIONS: The OMT classification can be used to describe hand anomalies that may present in over 1,400 diseases. CulaPhen was developed to provide a (hand) phenotype-based differential diagnosis. Differential diagnosis formulation based on the proposed system outperforms the system in current use.

CLINICAL RELEVANCE: This study illustrates that the OMT diagnoses, either individually or combined, can be cross-referenced with different diseases and syndromes. Therefore, use of the OMT classification can aid differential diagnosis formulation for CULA patients.

INTRODUCTION

Congenital upper limb anomalies (CULA) are developmental disorders, which can occur in isolation, as one of the features of an association or as a part of a syndrome[1,2,75]. Although many present as an isolated malformation, severe comorbidities and even perinatal death may result from associated anomalies[75] in syndromal cases. Thus the surgeon is not only confronted with the difficulty of managing a child with a rare anomaly, but should also consider the risk of additional malformations related to the hand anomaly.

CULA exhibit a wide spectrum of anomalies, each of which might be related to different diseases. Therefore, the structure provide by the newly accepted Oberg, Manske, and Tonkin (OMT) classification could aid in formulating a differential diagnoses in patients with an OMT-driven system, CulaPhen, which enables the clinician to obtain a differential diagnosis of diseases and genes that may be associated with the observed

phenotype in patients with CULA. CulaPhen has been validated by comparison with current literature and the CULA[1,2,5,21]. Furthermore, because the OMT classification was designed using the current understanding of embryology, it could also provide insight into anomalies and malformations that may be related[5,20].

Differential diagnosis in developmental disorders can be based on databases such as the Online Mendelian Inheritance in Man (OMIM)[76], Orphanet[77], Possum[78] and the London Dysmorphology Database[79] all of which serve as reference work for clinicians. However, these databases are disease-based and often have little emphasis on the different types of CULA. These databases are hard to use when considering just the observed hand anomaly and are therefore not ideal for the clinician dealing with CULA.

The Human Phenotype Ontology (HPO) project[80] aims to provide a structured phenotypic framework of all anomalies in Mendelian diseases. Unfortunately, the HPO project describes CULA using terms derived from the literature or from other databases, which do not necessarily correlate with the OMT classification and its embryological foundation. For example, the HPO regards hypoplastic thumb and radial polydactyly as two different entities namely “Aplasia/hypoplasia of digits” and “Polydactyly”. However, the OMT classification places both malformations in the same class since both are disruptions of the radial-ulnar axis in hand plate formation. The use of universal nomenclature by all specialists, as is provided by the OMT classification, may prevent misinterpretation of phenotypes and ultimately the misdiagnosis of diseases.

The first aim of this study was to review the HPO database for all hand phenotypes in the OMT classification and subsequently derive all related diseases and genes. The second aim was to provide insight into the presentation of these diseases by systematically collecting and classifying all related phenotypes and inheritance patterns. The overall goal was to produce an OMT driven system, CulaPhen, which enables the clinician to obtain a differential diagnosis of diseases and genes which can be associated with the observed phenotype in patients with CULA. CulaPhen has been validated by comparison with current literature and the current HPO disease prediction tool, Phenomizer[81].

METHODS

Culaphen methodology

The CulaPhen dataset was developed based on the monthly release of the HPO dataset, the HPO source file, which contains all diseases for which a gene was located, and all contributing phenotypes. More information regarding the release and content is presented in Appendixes A-C.

Identification of diseases which can present with hand anomalies:

To derive all diseases that can present with hand anomalies, all unique HPO phenotypes were extracted from the HPO source file and categorized into anatomical groups based on the Rotterdam registration form for congenital upper anomalies[8] (12 groups: CULA, Circulatory, Respiratory, Digestive, Urogenital, Nervous System, Vertebral Column, Musculoskeletal, Head/Neck, Lower limb, Skin, Others). An additional group (group 0) was created from the HPO source file to describe the inheritance patterns for each disease. A subset of the HPO source file was made including only diseases with at least one hand anomaly in the phenotypic description of the disease.

Classification of hand phenotypes:

All HPO phenotypes in the CULA group were classified by 2 of the authors (MB and C.A.v.N.) according to the latest modification of the Oberg, Manske and Tonkin classification[5]. The HPO phenotypes with unclear terminology were reviewed in the HPO browser to obtain a full description of the term to clarify the phenotype. When needed, the gene and disease relations for that HPO phenotype were reviewed to clarify the occurrence of the described HPO phenotype. The hand anomalies which did not fit the OMT classification, were either classified upon class level (such as the term “Carpal bone hypoplasia“). They were excluded when ambiguous (such as “Enlargement of wrists”), non-discriminating (such as “Abnormality of the Hand”) or hand anomalies which were “non-congenital” when rightfully not present in the OMT classification (such as “Drumstick terminal phalanges”), this state was verified by the consensus of both senior authors (C.A.v.N. and S.E.R.H.).

Disease presentation according to the anatomical groups:

For each disease, the number of HPO phenotypes among the 12 anatomical groups was counted and expressed in a ratio reflecting the contribution of that anatomical group to the total number of phenotypes known for that disease. Subsequently, the maximum score for one of the anatomical groups equals 1, indicating that 100% of the known HPO phenotypes for that disease is in that specific anatomical group. If the ratio equaled 0, then no HPO phenotype is present in the anatomical group of interest. The concept of this ratio of contribution to a disease and its visualization is illustrated in Figure 1.

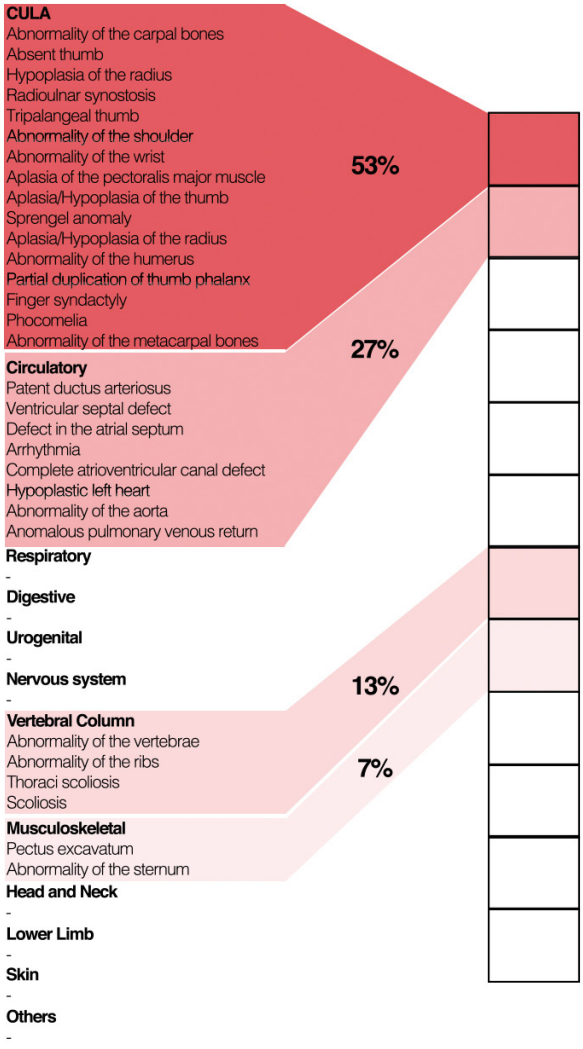


Figure 1. Heat map visualization of the ratio of contribution of the 12 different anatomical groups, using Holt-Oram syndrome (OMIM: 142900) as an example. According to the HPO database, Holt-Oram can present with (a combination of) 30 different unique phenotypes. The 30 different unique phenotypes were clustered in 12 different anatomical groups: 16 (53%) of those phenotypes fall into the CULA category, 8 (27%) phenotypes fall into the Circulatory category and 4 (13%) and 2 (7%) phenotypes fall into the Vertebral Column and Musculoskeletal category. The ratio of contribution of the phenotype to a disease is translated to a shade of red, from white (0%) to bright red (100%), creating a disease specific heat map.

Heat map visualization:

Heat maps are commonly used to visually depict a measure of association between 2 variables. In CulaPhen, heat maps are used to depict the relation between a disease and a group of associated anomalies, as measured by the ratio of contribution. Heat map visualization of the CulaPhen dataset was performed using Spotfire (version 7,TIBCO Spotfire Inc., CA, USA). To create a heat map, one or more OMT diagnoses can be selected, with or without a mode of inheritance. When multiple OMT diagnoses are selected, only the diseases for which all diagnoses have been reported will be visualized. The heat map visualizes the ratio of contribution of all 12 anatomical groups to each disease. The x-axis depicts the different diseases with their causal genes, whereas the y-axis depicts the 12 different anatomical groups. A color gradient from 0 (white) to 1 (bright red) was used to visualize the ratio of contribution, where white indicates no HPO phenotypes present in a given anatomical group and a bright red indicates all (100%) of the HPO phenotypes known for this disease are present in a given anatomical group.

Differential diagnosis:

Differential diagnosis formulation can be achieved using the CulaPhen TIBCO Spotfire application. By selection of the anatomical groups of interest (e.g. the affected anatomical groups observed in a patient), diseases will be selected with a ratio of contribution higher than 0% for each individual groups. A sum of the individual ratios for each of the selected groups will be calculated to express what percentage of phenotypes for each given disease is in the selected groups. Subsequently the diseases will be ranked upon this sum (highest through lowest score). In addition, a web link is provided for each disease in the selection to either the Orphanet or the OMIM database as reference.

Validation of hand phenotypes

Because many case reports on hand anomalies and disease or gene associations are published each year, it is of imminent importance the source data is up-to-date. To illustrate that the obtained diseases and their phenotypic description were up-to-date, in July 2015 the latest 10 (case) reports describing anomalies of the upper limb and a genetic locus filed were selected. Only case studies indexed under the MESH term “limb deformities, congenital/genetics” in PubMed/MEDLINE which were online available and in English were considered. The described phenotypes were extracted and referenced to the OMT dataset and assessed for presence of the relation, content of the relation (different hand phenotypes) and known other aberrations.

Validation of disease predication based on the observed phenotype (Case Studies)
To demonstrate the clinical application of the tool, anonymized example cases were obtained from the Rotterdam database of congenital anomalies. This database was built under approval of the local medical ethics committee (MEC-2015-012). All diagnosis of case examples were confirmed with a molecular diagnostics.

Hand phenotypes and associated anomalies were used to provide a CulaPhen output for each case example were compared with the Phenomizer[81] predicted phenotype. Phenomizer uses unedited HPO datasets and creates a differential diagnosis based on a composed similarity score. Since Phenomizer is built using the HPO project then this application was regarded the golden standard for our study. The output from both CulaPhen and Phenomizer were compared on rank number of the molecularly confirmed disease and related gene and since Phenomizer also includes diseases with no known genetic cause, these diseases were excluded from our comparison.

RESULTS

Culaphen methodology

A total of 514 CULA were identified from all 5877 HPO phenotypes. When placed in the OMT classification, 319 hand phenotypes described a malformation, two described a deformation and 63 described a dysplasia of the upper limb. A total of 130 upper limb related HPO phenotypes were excluded, 11 due to ambiguous terminology, 22 terms were labeled non-congenital at second review, 58 terms were non-discriminating (could match numerous OMT classes) and finally 39 terms were not classifiable. Appendix D lists excluded phenotypes and the motivation for exclusion. Of 4,472 diseases from the HPO dataset, 3,069 were excluded because the phenotypic description of these diseases did not include a hand anomaly. This resulted in a selection of 1,403 hand-related diseases and 1,204 hand-related genes that could be linked to the OMT classification. Details on the creation of the relationships, including the date of extraction of source data, the number of excluded non-hand phenotypes, and the OMT terms with the largest number of HPO phenotypes are available in Appendixes A to C.

The number of hand phenotypes described for each gene was widely variable, from one to 82 hand phenotypes for one gene (median=2; 25th percentile=1; 75th percentile=5). Table 1 lists the distribution of HPO phenotypes, related diseases, and genes among the classes of the OMT classification.

Of the 12 anatomical groups, head and neck anomalies had the largest overall contribution to the selected diseases (22%), whereas respiratory symptoms or anomalies were least common (1.8%). The overall median contribution of the anatomical groups to a dis- ease was 4.4% (range, 0% to 100%; interquartile ranges, 0.0% [25th percentile] to 11.6% [75th percentile]).

Figure 2 illustrates visualization and use of the obtained data by the selection of 2 OMT diagnoses.

Table 1. Number of phenotypic identifiers, associated diseases and associated genes for each class of the OMT classification

Classes in the OMT classification		Matching HPO terms	Associated diseases	Associated genes
1. Malformations		319	1226	1075
1A. Upper limb		96	520	551
1A1. Prox-distal		22	154	155
1A2. Rad-ulnar	45	165	174	
1A3. Dors-ventral	5	4	4	
1A4. Unspecified	22	293	344	
1B. Hand plate		231	1013	862
1B1. Prox-distal		71	354	310
1B2. Rad-ulnar	35	234	216	
1B3. Dors-ventral	37	258	247	
1B4. Unspecified	85	655	618	
1B4a. Soft tissue	33	510	483	
1B4b. Skeletal	41	234	256	
1B4c. Complex	19	198	213	
2. Deformations		2	8	9
2A. Constriction ring		1	7	8
2C. Trigger digit		1	1	1
3. Dysplasia		63	203	223
3A. Hypertrophy		5	10	16
3A1. Whole limb		2	5	9
3A2. Partial limb		3	7	9
3B. Tumor		58	196	212
3B1. Vascular		3	27	46
3B4. Skeletal		54	173	170

Table 1

Table 1 displays the number of phenotypic identifiers, associated diseases and associated genes for each class of the OMT classification. Most diseases have been described within Malformations, especially malformation of the hand plate. An overlap in disease associations can be detected, while the total number of diseases is lower than the sum of the individual OMT-classes.

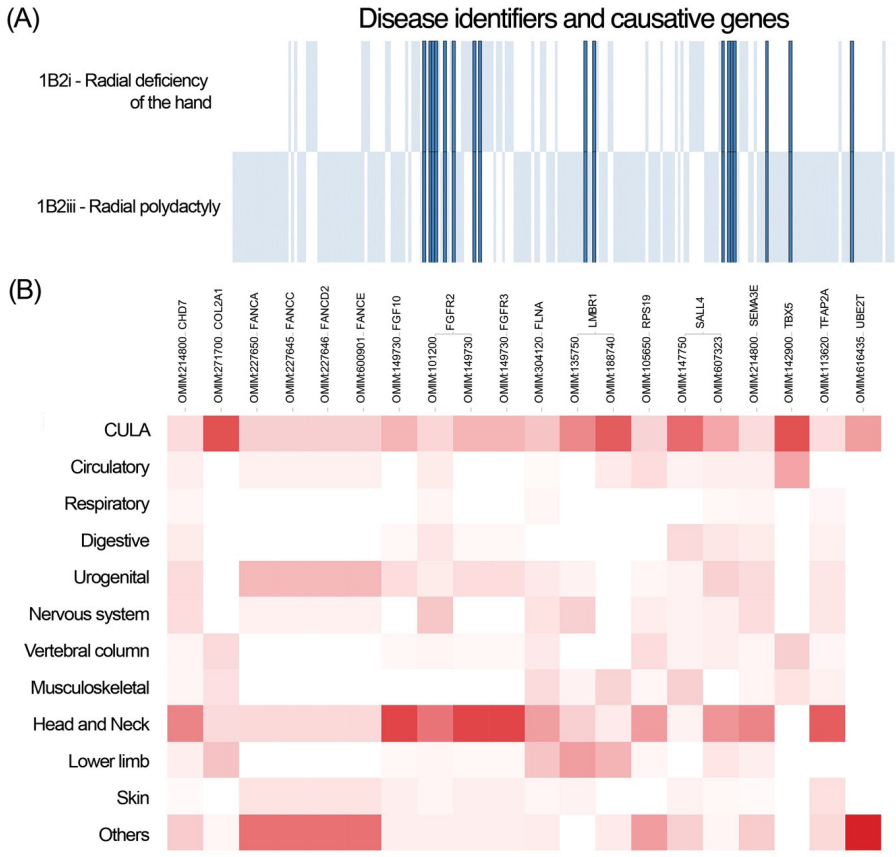


Figure 2 shows the successful differentiation between diseases in which both radial deficiency of the hand and radial polydactyly can occur. (A) Shows all individual diseases and causative genes with a relation to either "1B2i-Radial deficiency of the hand" and "1B2iii-Radial polydactyly". Contiguous dark dark blue lines indicate that both phenotypes are present in that particular disease. Light blue blocks indicate that that phenotype is present in the disease, however one of the other phenotypes is not present. White blocks indicate that neither of the phenotypes are present for the disease. (B) All diseases for which both phenotypes are present were visualized using a heat map. Using the heat map visualization of the ratio of contribution allows for further differentiation: e.g. based the prominent contribution of head and neck anomalies in all *FGFR*- related diseases as compared to OMIM:142900 for which no head and neck anomalies are present. In the tool, the value of this ratio of contribution can be used for disease ranking.

Validation of hand phenotypes

The validation set consisted of the 10 recently published hand phenotypes were identified from litrature[82-91]. In 8 out of 10 cases, the presented hand phenotypes matched the OMT diagnoses in our dataset using the CulaPhen methodology (Table 2). All causative genes related to the published phenotypes were available in our dataset. In the other 2 cases (*TGD5* and *BHLHA9*) a hand with multiple different anomalies was presented, which did not completely match the OMT diagnoses in CulaPhen. The nature of the presented associated aberrations matched the grouped phenotypes in all, except for the respiratory symptoms described by Mitsui et al. (apnea in PGE4D case).[87]

Validation of disease prediction based on the observed phenotype

The CulaPhen output provided for 2 family examples in figure 3 and 4 shows how the clinical features of the presented phenotypes provide valuable differentiation between diseases. A short description of the output interpretation is available in the figure legend, detailed explanation on usage of the output is provided in Appendix E. Overall performance for 2 families and 3 cases is provided in table 3. In 4 out of 5 cases, CulaPhen obtained a higher rank for disease and gene targets as compared with Phenomizer.

Table 2. Comparison phenotypes presented in 10 recent reports on congenital upper limb anomalies to the contents of the CulaPhen dataset.

Author	Year	Gene	Gene present	Hand phenotype in literature	Found hand phenotypes in CulaPhen	Other aberrations in literature	Category in CulaPhen
Stittrich et al.	2014	NOTCH1	Yes	Terminal transverse limb defects	1B1iii. Transverse deficiency of the hand	Aplasia cutis	Skin
					1B1i. Brachydactyly	Cutis Marmorata	
					1B3ii. Ventral dimelia (incl hypoplastic/aplastic nail)	Intracranial vascular lesions	Nervous
					1B4ia. Syndactyly	pulmonary hypertension	Respiratory
					1B4iiic. Cleft Hand	Cardiac malformation	Circulatory
Sowinska-Seidler et al.	2014	DLX5	Yes	Split hand	1B4iiic. Cleft Hand	Split foot	Lower limb
					1B1iii. Transverse deficiency of the hand		
					1B3ii. Ventral dimelia (incl hypoplastic/aplastic nail)		
					1B4ia. Syndactyly		
					1B4iiia. Complex syndactyly		
Ehmke et al.	2014	TGDS	Yes	Manzke dysostosis	1B4ib. Camptodactyly	Congenital heart defect	Circulatory
				Clinodactyly	1B4iia. Clinodactyly	Pierre Robin sequence	Head Neck
				Brachymetacarpia	3B4iv. Epiphyseal anomalies	Facial dysmorphisms	
				Adducted thumbs	Growth retardation Hearing loss	Cleft Palate	
				Short humerus		Other	

Author	Year	Gene	Gene present	Hand phenotype in literature	Found hand phenotypes in CulaPhen	Other aberrations in literature	Category in CulaPhen
Irfan Raza et al.	2015	PVRL4	Yes	Cutaneous syndactyly	1B4ia. Syndactyly	Alopecia	Skin
				Abnormalities of the nail	1B3ii. Ventral dimelia (incl hypoplastic/aplastic nail)	Deformed pinnae ears	Head
						Syndactyly of the feet	Lower limb
						Abnormalities of the toenails	
White et al	2015	DVL1	Yes	Mesomelia	1A1i. Brachymelia	Numerous facial features like: macrocephaly and frontal bossing	Head Neck
					1A1iv. Intersegmental deficiency		
				Brachydactyly	1B1i. Brachydactyly		
				hypoplastic phalanges		Pectus anomaly	Musculo-skeletal
				Clinodactyly	1B4iia. Clinodactyly	Umbilical hernia	
				Camptodactyly	1B4ib. Camptodactyly	Scoliosis	Vertebral
				Broad/duplicated thumb	1B2iii. Radial Polydactyly	Broad first toe	Lower limb
				Nail dysplasia	1B3ii. Ventral dimelia (incl hypoplastic/aplastic nail)	Urogenital features	Urogenital
					1B4ia. Syndactyly	Heart defects	Circulatory
					1B4iiia. Complex syndactyly	Seizures	Nervous
					1A2v. Congenital radial head dislocation		

Author	Year	Gene	Gene present	Hand phenotype in literature	Found hand phenotypes in CulaPhen	Other aberrations in literature	Category in CulaPhen
Mitsui et al	2014	PDE4D	Yes	Stubby digits	1B1i. Brachydactyly	Short stature	Other
					1A1i. Brachymelia	Hypoplastic nasal root	Head Neck
					1B3ii. Ventral dimelia (incl hypoplastic/aplastic nail)	Depressed nasal tip	
					3B4iv. Epiphyseal anomalies	Short philtrum	
					1A2i. Radial longitudinal deficiency	Down-turned mouth	
						iris heterochromia	Other
						tibial bowing	Lower limb
						intellectual impairment	Nervous
						muscle weakness	
						Apnea	**NOT PRESENT**
						failure to thrive	Other

Author	Year	Gene	Gene present	Hand phenotype in literature	Found hand phenotypes in CulaPhen	Other aberrations in literature	Category in CulaPhen
Malik et al.	2014	BHLHA9	Yes	Mexoaxial synostotic syndactyly of the Malik-Percin type 1B4iiia. Complex syndactyly	1B4ia. Syndactyly mesoaxial toe webbing	pre-axial fusion of toes	Lower Limb
				*synostosis at metacarpal level	1B4iib. Synostosis/symphalangism		
				*adactyly	1B1iii. Transverse deficiency of the hand		
				*hypoplasia of distal phalanges post and pre-axial 1B2i. Radial deficiency of the hand	1B1i. Brachydactyly		
				*clinodactyly of fifth finger	1B4iia. Clinodactyly		
					1B2iii. Radial Polydactyly		
					1B2vi. Ulnar polydactyly		
					1Biiic. Cleft Hand		
Reda Belkhribchia et al.	2014	SOST	Yes	Radial deviation of terminal phalanges	1B4iia. Clinodactyly	Bilateral facial paresis	Head Neck
				2nd finger	1B3ii. Ventral dimelia (incl hypoplastic/aplastic nail)	Syndactyly of the feet	Lower limb
				1B4iia. Clinodactyly 1B4iiia. Complex syndactyly	1B4ia. Syndactyly Thickened cortices	dysplastic nails Musculoskeletal	

Author	Year	Gene	Gene present	Hand phenotype in literature	Found hand phenotypes in CulaPhen	Other aberrations in literature	Category in CulaPhen
Nandagopalan et al.	2014	PRG4	Yes	Contractures of the metacarpophalangeal and interphalangeal joints 1B1i. Brachydactyly 1B4ib. Camptodactyly	1B4id. Distal arthrogryposis Swelling of the joints	Osteopenia	Musculo-skeletal
Sajid Hussain et al.	2014	CKAP2L	Yes	Syndactyly	1B4ia. Syndactyly	Ectodermal abnormalities	Skin
				Clinodactyly	1B4iia. Clinodactyly	Syndactyly of the feet	Lower limb
				Hypoplasia of phalanges	1B4iiia. Complex syndactyly	Facial dysmorphisms	Head Neck
						Neurological aberrations	Nervous

Table 2

Table 2 shows the review of the 10 recent case reports indexed under MESH term "limb deformities, congenital/genetics", indicating whether the gene relation was available in our tool, the relation of reported anomalies in the case report and the data in the tool. In the studies by Ehmke et al. and Malik et al. a complex anomaly was described, which was not (completely) classifiable using the hand phenotypes in CulaPhen. *Matching phenotypes in the OMT classification. ⁺partially matching OMT diagnosis.

Table 3. Rank number comparison of gene and disease prioritization in 5 case/family examples using CulaPhen and Phenomizer.

Case	CulaPhen		Phenomizer		Patient features used for analysis
	Gene	Disease	Gene	Disease	
Family 1 (Greig syndrome, GLI3)	#2	#3	#7	#7	Radial polydactyly (hand and feet), ulnar polydactyly, syndactyly, hypertelorism (dominant)
Family 2 (MASA syndrome, L1CAM)	#1	#1	#1	#1	Thumb in palm deformity, camptodactyly, hydrocephalus, scoliosis (recessive)
Case 3 (Holt-Oram, *TBX5*)	#1	#1	#25	#25	radial polydactyly, triphalangeal thumb, atrial and ventricular septal defect (all modes of inheritance)
Case 4 (Brachydactyly, GDF5)	#3	#3	#24	#24	Brachydactyly type C (all modes of inheritance)
Case 5 (Synpolydactyly 1, HOXD13)	#1	#1	#10	#10	Syndactyly of hand, postaxial polydactyly feet (all modes of inheritance)

Table 3

Table 3 displays observed rank of the clinically diagnosed disease and related gene from both CulaPhen and Phenomizer considering the gene and disease of interest. In Culaphen, the affected organ group (e.g. Circulatory in Holt-Oram syndrome) was used for prioritization, in Phenomizer the exact phenotype was used (e.g. VSD in Holt-Oram syndrome). In Family 1 and Cases 3-5 CulaPhen had a better rank than Phenomizer. In Family 2, the same results were obtained. In both families and in all presented cases, CulaPhen produced a differential diagnosis in which the observed syndrome or gene was in the top 3 choices. In Phenomizer, this was only the case for Family 2.

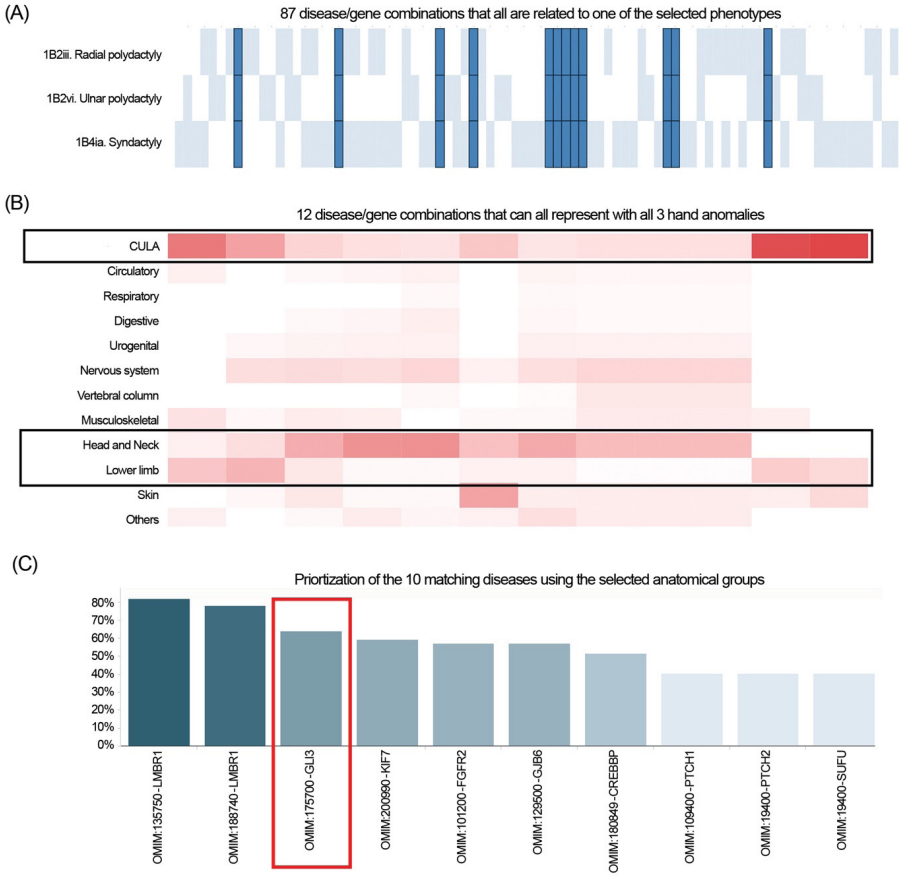


Figure 3 shows how CulaPhen helps the clinician in decision making for Family 1. In this family radial and ulnar polydactyly, syndactyly, hypertelorism and duplicated halluces were observed. Hence, the affected organ groups for prioritization were: CULA, Head and Neck and Lower Limb. As both parents and children were affected, a dominant inheritance pattern was assumed. **(A)** Figure 3a. shows the 87 diseases are known with this inheritance pattern and either one of the hand phenotypes. Contiguous dark dark blue lines indicate that all 3 phenotypes are present in that particular disease. Light blue blocks indicate that that phenotype is present in the disease, however one of the other phenotypes is not present. White blocks indicate that the phenotype is not present for that disease. Only 12 diseases are known with the combination of hand phenotypes. **(B)** The heat map visualization is used to display the ratio of contribution of each anatomical group to the different diseases. The black boxes indicate the anatomical groups used for prioritization. (**C)** Using sum of the ratio of contribution for the selected anatomical group, it can be determined that the combination of observed associated anomalies matches OMIM:188740 (Tibia hypo/aplasia with polydactyly) the best (82%). The family was diagnosed with Greig syndrome, which is the 3rd ranked disease and the 2nd ranked gene in this analysis.

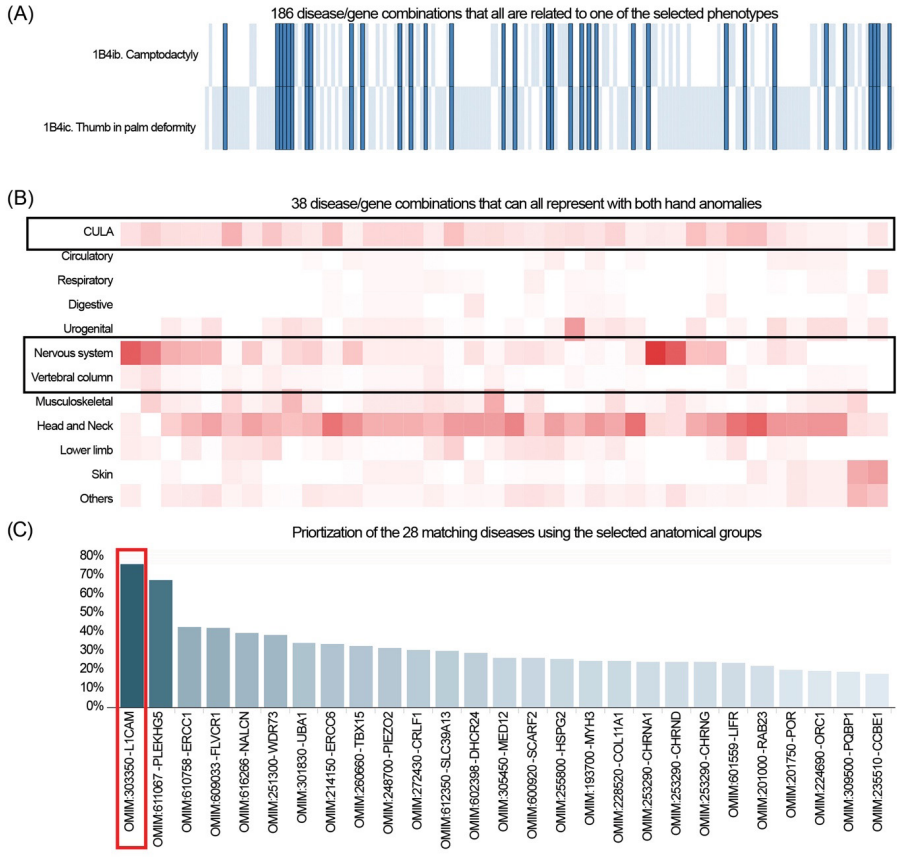


Figure 4 shows how CulaPhen helps the clinician in decision making for Family 2. In this family, thumb in palm deformities, camptodactyly, mental retardation, hydrocephalus and scoliosis was observed in two brothers. Hence, the affected organ groups for prioritization were: CULA, Nervous system and Vertebral Column. The assumed inheritance was either autosomal recessive or X-linked since both parents were unaffected. (A) In total 234 diseases match the assumed inheritance pattern and one of the hand phenotypes. Contiguous dark dark blue lines indicate that all 3 phenotypes are present in that particular disease. Light blue blocks indicate that that phenotype is present in the disease, however one of the other phenotypes is not present. White blocks indicate that the phenotype is not present for that disease. However, the combination of hand phenotypes is only found in 38 possible diseases. (B) The heat map visualization is used to display the ratio of contribution of each anatomical group to the different diseases. The black boxes indicate the anatomical groups used for prioritization. (C) Figure 4c shows the combined ratio of contribution of the selected anatomical groups. Diseases are sorted on the highest score (left to right). The combination of CULA, neurological diseases and vertebral anomalies can best be described by OMIM:303350 (MASA syndrome). This family was diagnosed with MASA syndrome (rank 1), an *L1CAM* (rank 1) related disease.

DISCUSSION

The OMT classification was introduced to replace the IFSSH/Swanson classification[92] and its publication was succeeded by multiple studies assessing its use to classify observed hand anomalies. However, the OMT classification also aims to cross-reference anomalies with syndromes not reported in any of the previous studies[1,2,5,21]. In this study, we used the HPO dataset to review the relation of over 1400 Mendelian diseases to the OMT classification. In doing so, we illustrate that the proposed cross-reference of OMT diagnoses with syndromes is possible and actually provides the basis for better differential diagnosis formulation for CULA patients. CulaPhen enables the clinician to make an accurate differential diagnosis using both the observed hand anomalies and other observed congenital malformations.

In the CulaPhen methodology, the associated anomalies are of importance to the evaluation of the disease presentation and finally to formulate a differential diagnosis. Although evaluating associated anomalies might not be daily practice for all hand surgeons, its importance has been stressed in multiple studies; e.g. up to 18% of all CULA cases have been reported to die in the first six years in an Australian population, mainly because of these associated anomalies[3]. Therefore, a structured description of associated anomalies, as for example used in the Rotterdam form for CULA[8] was advocated over a decade ago. The CulaPhen methodology emphasizes yet again that this data is actually valuable for the patient in delivering an accurate differential diagnosis.

To demonstrate that the source data used for CulaPhen is up to date, we studied the hand phenotype and compared this to the available OMT-terms in CulaPhen for the observed genetic cause in 10 current case reports on hand anomalies and their genetic cause selected from literature. All causative genes described in the case report were present in CulaPhen and in 8 out of 10 cases the observed anomalies matched the OMT description in CulaPhen. However, complex or compound phenotypes such as presented in the article by Malik et al.[88] are not easily recognizable in the source dataset. This could hinder proper differential diagnosis formulation, as can also be illustrated using Apert syndrome as an example. The hand phenotype found in Apert syndrome can include complex syndactyly, (meta)carpal synostosis, symphalangism and ulnar or radial polydactyly, however this description fails to identify the unique entity referred to as “Apert hand” in the OMT classification. The individual hand anomalies can be observed in numerous diseases, whereas the unique combination is limited to Apert syndrome. However, as Flottman et al.[93] illustrate, we should also consider the diagnosis of severe syndromes in mildly affected patients with only a few of the key features of a syndrome. Therefore, the compound phenotypes were not replaced with their “umbrella” term.

The clinical examples in this article illustrate that CulaPhen is as good as or superior to Phenomizer[81] in the prediction of diseases based on the hand anomaly (including the observed associated anomalies). In both familial examples and isolated cases, CulaPhen managed to produce a differential diagnosis with the actual disease of interest in the top 3 of the differential diagnosis. When compared to Phenomizer, the improved prioritization might save a clinician or researcher up to 24 diseases to evaluate (Table 3, Holt-Oram syndrome, rank 1 vs. rank 25).

Besides the clinical application of CulaPhen, there also is a research purpose of the tool. In his personal opinion published in the European Journal of Hand Surgery, Al-Qattan[94] stresses the importance of the involvement of both (clinical) geneticists and hand surgeons in genetic research to identify new diseases and gene relations[95,96]. Our methodology and tool provide a first step to get involved with genetic research, as it provides a fast overview of known diseases and genes by means of the OMT classification. Furthermore, it easily connects to its sources like OMIM and Orphanet for extended research on the syndrome and related publications. Therefore, it can be of great benefit for those surgeons interested in genetic research.

The success rates of conventional genetic research in CULA patients are low, however the application of next generation sequencing (NGS) could improve these rates. Furniss et al. even argued that *GLI3, HOXD13* and the regulatory region of *SHH* (ZRS) should be the only genomic targets addressed in primary genetic screening since these were the only 3 affected in the 202 individuals screened using a total of 12 genomic targets[66]. However, the solved cases in their analyses were all from a particular spectrum of hand anomalies: 65% (21/32) of the solved in their study had hand anomaly caused by a disruption of the radio-ulnar axis (OMT classification). Therefore, their recommendation might not hold for all anomalies present in the OMT classification. NGS allows the clinician to target all human genes, however subsequently introduces the necessity to filter and prioritize the results from these experiments. CulaPhen provides a hierarchically ordered number of related genes and diseases given the observed hand phenotypes. Therefore, future research should evaluate the prioritization provided by CulaPhen in next-generation sequencing.

In conclusion, we present an OMT based overview of Mendelian diseases and related genes that can present with congenital upper limb anomalies. Furthermore, we aid the clinician in differential diagnosis formulation and decision making by providing insight in the disease presentation. The data from our research is freely available for use in our CulaPhen application. CulaPhen is up to date and outperforms current tools for disease and gene prioritization in patients with congenital upper limb anomalies.

APPENDIX A

Input file 12-10-2015: ALL_SOURCES_ALL_FREQUENCIIES_diseases_to_genes_to_phenotypes.txt
Total number of relations: 96107
Total number of diseases: 4472
Total number of genes: 3248
Total number of phenotypes: 5877
Hand related relations: 5858
Hand related diseases: 1403
Hand related genes: 1204
Filtered non-hand related diseases: 3069

APPENDIX B

	Unique HPO identifiers	Total used in source dataset	Hand Subset total	
CULA	514	5843	5843	1.0
Circulatory	296	3492	1932	0.55
Respiratory	161	2105	1141	0.54
Digestive	260	4246	2057	0.48
Urogenital	392	5647	3384	0.60
Nervous system	737	16612	8190	0.49
Vertebral column	156	1794	1519	0.85
Musculoskeletal	430	5455	3997	0.73
Head/Neck	828	22177	14705	0.66
Lower limb	477	3132	2820	0.90
Skin	358	4288	2687	0.63
Others	1407	15360	6517	0.42

Number of unique phenotypic identifier, the total number of associations with the phenotypes in the total set and the subset (both absolute and relative) for each group of aberrations

APPENDIX C

OMT Diagnosis	OMT code	HPO terms
Brachydactyly	1B1i	67
Epiphyseal abnormalities	3B4iv	50
Aberrant/a-plastic nails	1B3ii	37
Clinodactyly	1B4bi	23
Phalangeal / Carpal / Metacarpal synostosis	1B4biii	18
Arthrogryposis / Windblown hand	1B4aiv	14
Ulnar longitudinal deficiency	1A2ii	11
Syndactyly	1B4ai	11
Camptodactyly	1B4aii	11
Radial longitudinal deficiency	1A2i	10

Top 10 OMT diagnoses with class codes and the number of matching HPO terms

APPENDIX D

TERM UNIQUE TO HPO IDENTIFIER	Reason for exclusion
Crowded carpal bones	Ambiguous terminology
Enlargement of the wrists	Ambiguous terminology
Increased carrying angle	Ambiguous terminology
Irregular carpal bones	Ambiguous terminology
Irregular metacarpals	Ambiguous terminology
Large hands	Ambiguous terminology
Limited elbow movement	Ambiguous terminology
Limited interphalangeal movement	Ambiguous terminology
Limited mobility of proximal interphalangeal joint	Ambiguous terminology
Limited wrist movement	Ambiguous terminology
Small hand	Ambiguous terminology
Chondrocalcinosis	Non-congenital
Clubbing	Non-congenital
Clubbing of fingers	Non-congenital
Cold-induced hand cramps	Non-congenital
Dagger-shaped pulp calcifications	Non-congenital
Dislocated wrist	Non-congenital
Drumstick terminal phalanges	Non-congenital
Edema of the dorsum of hands	Non-congenital
Enlarged interphalangeal joints	Non-congenital
Enlarged joints	Non-congenital
Enlarged metacarpophalangeal joints	Non-congenital
Enlarged proximal interphalangeal joints	Non-congenital
Fingerpad telangiectases	Non-congenital
First dorsal interossei muscle atrophy	Non-congenital
Hand muscle atrophy	Non-congenital
Heberden's node	Non-congenital
Interphalangeal joint erosions	Non-congenital
Metacarpal diaphyseal endosteal sclerosis	Non-congenital
Patchy sclerosis of finger phalanx	Non-congenital
Swelling of proximal interphalangeal joints	Non-congenital
Weakness of the intrinsic hand muscles	Non-congenital
Wrist swelling	Non-congenital
Abnormal calcification of the carpal bones	Non-discriminating
Abnormal diaphysis morphology	Non-discriminating

TERM UNIQUE TO HPO IDENTIFIER	Reason for exclusion
Abnormal finger flexion creases	Non-discriminating
Abnormal hand bone ossification	Non-discriminating
Abnormal ossification of hand bones	Non-discriminating
Abnormality of digit	Non-discriminating
Abnormality of finger	Non-discriminating
Abnormality of limb bone morphology	Non-discriminating
Abnormality of the carpal bones	Non-discriminating
Abnormality of the distal phalanx of finger	Non-discriminating
Abnormality of the elbow	Non-discriminating
Abnormality of the forearm	Non-discriminating
Abnormality of the hand	Non-discriminating
Abnormality of the humeroulnar joint	Non-discriminating
Abnormality of the humerus	Non-discriminating
Abnormality of the metacarpal bones	Non-discriminating
Abnormality of the metaphyses	Non-discriminating
Abnormality of the shoulder	Non-discriminating
Abnormality of the thumb	Non-discriminating
Abnormality of the ulna	Non-discriminating
Abnormality of the upper limb	Non-discriminating
Abnormality of the wrist	Non-discriminating
Abnormality of thumb phalanx	Non-discriminating
Abnormally shaped carpal bones	Non-discriminating
Accelerated skeletal maturation	Non-discriminating
Accessory carpal bones	Non-discriminating
Advanced ossification of carpal bones	Non-discriminating
Asymmetric growth	Non-discriminating
Bowed humerus	Non-discriminating
Broad palm	Non-discriminating
Decreased palmar creases	Non-discriminating
Deep palmar crease	Non-discriminating
Delayed ossification of carpal bones	Non-discriminating
Delayed phalangeal epiphyseal ossification	Non-discriminating
Delayed skeletal maturation	Non-discriminating
Distal widening of metacarpals	Non-discriminating
Elbow flexion contracture	Non-discriminating
Flared humeral metaphysis	Non-discriminating

TERM UNIQUE TO HPO IDENTIFIER	Reason for exclusion
Hypoplastic distal radial epiphyses	Non-discriminating
Hypotrophy of the small hand muscles	Non-discriminating
Irregular ossification of hand bones	Non-discriminating
Malaligned carpal bone	Non-discriminating
Multiple palmar creases	Non-discriminating
Narrow palm	Non-discriminating
Phalangeal dislocations	Non-discriminating
Prominent fingertip pads	Non-discriminating
Prominent interphalangeal joints	Non-discriminating
Radial metaphyseal irregularity	Non-discriminating
Shoulder dislocation	Non-discriminating
Single transverse palmar crease	Non-discriminating
Small epiphyses of the phalanges of the hand	Non-discriminating
Stiff interphalangeal joints	Non-discriminating
Stiff shoulders	Non-discriminating
Stippled calcification in carpal bones	Non-discriminating
Thin metacarpal cortices	Non-discriminating
Two carpal ossification centers present at birth	Non-discriminating
Ulnar metaphyseal irregularity	Non-discriminating
Varus deformity of humeral neck	Non-discriminating
Carpal osteolysis	Not classifyable
Expanded metacarpals with widened medullary cavities	Not classifyable
Expanded phalanges with widened medullary cavities	Not classifyable
Finger joint hyperextensibility	Not classifyable
Finger joint hypermobility	Not classifyable
High axial triradius	Not classifyable
Hitchhiker thumb	Not classifyable
Hyperextensibility at wrists	Not classifyable
Hyperextensibility of the finger joints	Not classifyable
Hyperextensible hand joints	Not classifyable
Hypermobility of distal interphalangeal joints	Not classifyable
Increased laxity of fingers	Not classifyable
Long distal phalanx of finger	Not classifyable
Long fingers	Not classifyable
Long metacarpals	Not classifyable
Long palm	Not classifyable

TERM UNIQUE TO HPO IDENTIFIER	Reason for exclusion
Long phalanx of finger	Not classifyable
Long proximal phalanx of finger	Not classifyable
Long second metacarpal	Not classifyable
Metacarpal osteolysis	not classifyable
Metacarpophalangeal joint hyperextensibility	Not classifyable
Metaphyseal cupping of proximal phalanges	Not classifyable
Multiple carpal ossification centers	Not classifyable
Osteolytic defects of the distal phalanges of the hand	Not classifyable
Osteolytic defects of the phalanges of the hand	Not classifyable
Pointed proximal second through fifth metacarpals	Not classifyable
Progressive clavicular acroosteolysis	not classifyable
Proximal tapering of metacarpals	Not classifyable
Severe carpal ossification delay	Not classifyable
Slender distal phalanx of finger	Not classifyable
Slender finger	Not classifyable
Slender metacarpals	Not classifyable
Slender proximal phalanx of finger	Not classifyable
Swan neck-like deformities of the fingers	Not classifyable
Tapered distal phalanges of finger	Not classifyable
Tapering pointed ends of distal finger phalanges	Not classifyable
Tombstone-shaped proximal phalanges	Not classifyable
Wide tufts of distal phalanges	Not classifyable
Widened metacarpal shaft	Not classifyable

APPENDIX E

Family I presented with both radial and ulnar polydactyly and syndactyly. Furthermore, there was also a polydactyly of the hallux and hypertelorism. The family includes two affected brothers, whom both had children with the same phenotype, subsequently a dominant or X-linked dominant inheritance pattern was assumed. Individually, radial polydactyly, ulnar polydactyly and syndactyly have 116, 136 and 150 disease annotations respectively, taking in consideration the segregation pattern 59, 36 and 44 disease annotations remain.

Figure 3A shows all radial and ulnar polydactyly and syndactyly diseases and genes matching the inheritance pattern. Contiguous dark dark blue lines indicate that all 3 phenotypes are present in that particular disease. Light blue blocks indicate that that phenotype is present in the disease, however one of the other phenotypes is not present. White blocks indicate that the phenotype is not present for that disease. In total 12 diseases are known for which all 3 phenotypes.

Figure 3B. shows the involvement according to the ratio of contribution for each anatomical group. Since in our example the family was affected with hand anomalies, head and neck anomalies (hypertelorism) and anomalies of the feet (duplicated hallux) all data in the analysis is sorted on the combined ratio of contribution for those 3 anatomical groups. On the left-hand side OMIM:188740 (Tibia hypo/aplasia with polydactyly) is the first ranked disease, while the three anatomical groups contain 77,8% of all associated phenotypes to that disease. To illustrate, in disease OMIM:109400 also all three anatomical groups can be affected, however only 40% of the associated phenotypes are categorized in the specified 3 groups. On the right-hand side OMIM:174500 has the worst match while only 2 groups have a known association. Head and neck anomalies are not present in the database for this disease.

This family was actually diagnosed with a mutation in the GLI3 gene and were diagnosed with OMIM:175700 (Greig cephalopolysyndactyly syndrome). This is the 2nd ranked gene and the 3rd ranked disease.

In the Phenomizer tool HPO terms “Finger syndactyly” (HP:0006101), “Preaxial hand polydactyly” (HP:0001177), “Preaxial foot polydactyly” (HP:0001841), “Postaxial polydactyly” (HP:0100259) and “Hypertelorism” (HP:0000316) were chosen, also an autosomal dominant inheritance was selected. Greig cephalopolysyndactyly syndrome and GLI3 were ranked 7th (see table 3)

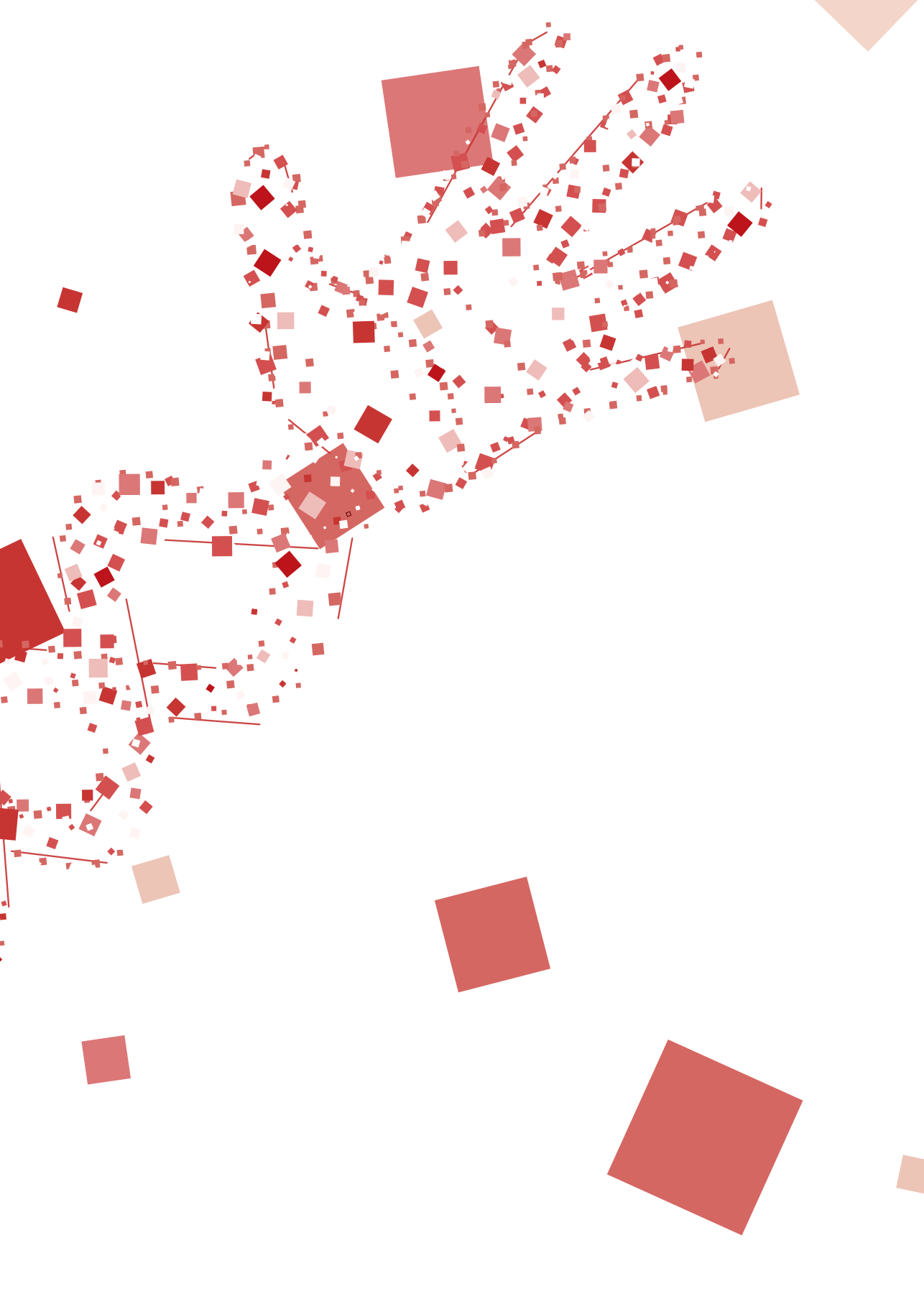
Family II consists of two brothers of unaffected parents present with thumb in palm deformities (both) and a camptodactyly (1 of the brothers). Both patients

suffered from hydrocephalus and mental retardation. One of the brothers also has a congenital scoliosis. Considering the parents were both not affected, a recessive or X-linked segregation was suspected.

Individually camptodactyly and thumb in palm deformity have 155 and 200 disease annotations respectively, considering the segregation pattern 88 and 136 diseases remained. Figure 4a shows that in total 38 different diseases can be recognized in which both camptodactyly and thumb in palm deformity have been described given the assumed inheritance. Contiguous dark dark blue lines indicate that all 3 phenotypes are present in that particular disease. Light blue blocks indicate that that phenotype is present in the disease, however one of the other phenotypes is not present. White blocks indicate that the phenotype is not present for that disease. In total 12 diseases are known for which all 3 phenotypes.

Figure 4B shows the involvement according to the ratio of contribution for each anatomical group. Since in our example the family was affected with hand anomalies, neurological conditions (hydrocephalus and mental retardation) and anomalies of the vertebrae (scoliosis) all data in the analysis is sorted on the combined ratio of contribution for those 3 anatomical groups. Again, on the left-hand side the disease with the largest combined ratio of contribution is displayed (OMIM:303350), in this case 75% of the known phenotypes were categorized in these three categories. The family in this example was in fact also diagnosed with MASA syndrome (OMIM:303350), an *L1CAM* associated disease. Both gene and disease were ranked first in this analysis.

In the Phenomizer tool HPO terms "Hydrocephalus" (HP:0000238), "Camptodactyly" (HP:0012385), "Scoliosis" (HP:0002650), "Adducted thumb" (HP:0001181) were chosen, in this analysis any inheritance pattern was regarded while Phenomizer does not allow multiple selection (both X-linked and autosomal recessive). Both *L1CAM* and MASA syndrome were listed first in the differential diagnosis produced by Phenomizer.



4

Controversies in Poland Syndrome: Alternative Diagnoses in Patients With Congenital Pectoral Muscle Deficiency

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ABSTRACT

PURPOSE: Poland syndrome was first described as a deficiency of the pectoral muscle with ipsilateral symbrachydactyly. Currently, numerous case reports describe variations of Poland syndrome, in which often only pectoral muscle deficiency is used as defining criterion. However, more syndromes contain pectoral muscle deficiency. The aim of this review is to study diversity of the phenotypic spectrum of Poland syndrome to create more awareness for alternative diagnoses in pectoral muscle deficiency.

METHODS: A systematic literature search was performed. Articles containing phenotypical descriptions of Poland syndrome were included. Data extraction included number of patients, gender, familial occurrence and the used definition of Poland syndrome. In addition, hand deformities, thoracic deformities, and other deformities were registered per patient. Alternative syndrome diagnoses were identified in patients with a combination of hand, thorax and other deformities.
RESULTS: Hundred-and-thirty-six articles were included, describing 627 patients. Ten different definitions of Poland syndrome were practiced. In 58% of the cases an upper extremity deformity was found, 43% had an associated deformity. Classical Poland syndrome was seen in 29%. Sixty percent of the patients with a pectoral malformation, a hand malformation and another deformity had at least one feature that matched an alternative syndrome.

CONCLUSIONS: Pectoral muscle hypoplasia is not distinctive for Poland syndrome alone but is also present in syndromes with other associated anomalies with a recognized genetic cause. Therefore, in patients with an atypical phenotype, we recommend considering other diagnoses and/or syndromes before diagnosing a patient with Poland syndrome. This can prevent diagnostic and prognostic errors.

CLINICAL RELEVANCE: Differentiating Poland syndrome from the alternative diagnoses has serious consequences for the patient and their family in terms of inheritance and possible related anomalies.

INTRODUCTION

The use of the term "Poland syndrome" has a long, controversial history in the literature. In 1841, Alfred Poland, described a cadaver with deficiency of the pectoral muscles and ipsilateral symbrachydactyly[97]. In 1895, Thomson was the first to document that syndactyly and deficiency of the pectoral muscles often accompany each other, which led to the suggestion of a new syndrome by Furst in 1900, characterized by deficiency of the pectoral muscles and ipsilateral syndactyly[98]. Two years later, Bing was the first to publish a case series of patients with deficiency of the pectoral muscles and syndactyly[99]. Nevertheless, it was 60 years later that Poland's name was used by Clarkson, a plastic and hand surgeon, as an eponym for the combination of deficiency of the pectoral muscle and syndactyly (Poland syndactyly)[98]. Unfortunately, the original phenotypic description of the patient of Alfred Poland was thereby abandoned. Subsequently, "Poland syndactyly" was transformed into "Poland syndrome" and its equivalents "Poland sequence" and "Poland anomaly". These terms have been used in scientific literature ever since[98].

Currently, the eponym Poland syndrome has become a universal term for clinicians to describe all disturbances of pectoral development, with or without symbrachydactyly. This is illustrated by Yiyit et al. who reported 113 patients with Poland syndrome of whom only 25 had various upper limb anomalies[100]. Moreover, Catena et al. described 8 different types of hand anomalies related to Poland syndrome[101]. The diversity of these reports raise the question of whether Poland syndrome is one entity or a group of separate sub-entities sharing only one phenotypic feature, namely pectoral deficiency.

Poland syndrome is not the only syndrome in which disturbances of pectoral development can be observed. For example, Holt-Oram and Duane-Radial-Ray syndrome both can present with absence of the pectoral major muscle together with upper limb anomalies[102,103]. Misdiagnosing patients with pectoral muscle deficiencies as Poland syndrome instead of one of the alternative diagnoses, might lead to false assumptions about etiology resulting in a failure to identify associated anomalies or genetic diagnoses.

To create more awareness on the alternative diagnoses in patients with pectoral muscle deficiency, we aim to illustrate the phenotypic spectrum of Poland syndrome in the literature by conducting a systematic review on its presentation. From this review, we identify all atypical Poland cases and define the phenotypic features that should alert the clinician for a possible alternative diagnosis. We hypothesize that the false use of the eponym Poland syndrome might result in misdiagnosis of some patients.

METHODS

For this systematic review, the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analysis) guidelines were followed and the checklist is available in the online supplements to this article (Appendix A). The systematic review protocol was registered in PROSPERO (CRD42015016679).

Search strategy

Seven different databases (Embase, Medline (OvidSP), Web-of-science, Scopus, Pubmed publisher, Cochrane, and Google scholar) were searched for eligible articles. The search strategies used are listed in Appendix B and the search was performed in May 2015. Original research articles and case reports containing a phenotypical description of Poland Syndrome in the Dutch or English language were included. Articles exclusively about treatment or surgery in Poland syndrome, Moebius syndrome, and general thoracic deformities were excluded. Moreover, reviews, letters-to-the-editor, and articles not available in full-text in the medical library of the Erasmus University Medical Centre, were also excluded.

Inclusion of articles was done by at least two out of three reviewers (M.B., E.B. and D.S.) and was based on screening of title and abstract. All differences between reviewers in the selection of articles were resolved by consensus. A subsequent exclusion of articles was done during full-text reading, when articles did not fulfil the inclusion criteria.

Data-extraction

Two reviewers (M.B. and E.B.) independently extracted data regarding study characteristics and outcomes with the use of a standardized extraction table. The included studies were scored based on number of patients, sex, familial cases and side of deformity. Furthermore, journal type, definition of Poland syndrome used in the paper, and causal hypothesis supported by the authors were extracted from the articles. A second database was created which included all separate patients described in the included studies. Specific hand and thoracic deformities were extracted, together with other reported anomalies and genetic outcomes. Other reported anomalies were classified in groups by cardiovascular, respiratory, urogenital, gastro-enteral, vertebral, neurological, craniofacial, dermatologic, oncologic, and other anomalies. Classical Poland cases, defined by a pectoral major muscle deficiency and ipsilateral symbrachydactyly, were also registered.

Alternative diagnoses

All syndromes with pectoral muscle involvement were extracted from the Human Phenotype Ontology (HPO) dataset[104]. The accession date and search terms are stated in Appendix B. The extracted syndromes were manually reviewed in the Online Mendelian Inheritance in Man (OMIM) database[97] to confirm pectoral muscle involvement combined with a hand anomaly. Furthermore, all other phenotypic characteristics of these syndromes were extracted from the OMIM database.

To evaluate whether the cases from the literature could fit an alternative diagnosis, cases with a combination of a pectoral malformation, a hand malformation, and an associated anomaly were selected. The selected cases were manually reviewed by the authors to check if any of the observed anomalies were concordant with an alternative diagnosis. The most commonly observed phenotypes, that could be related to an alternative diagnosis, were summarized.

RESULTS

Literature search results

Out of 1343 individual records returned by the initial search, 140 records describing a total of 948 patients were included[99-101,105-241] (Figure 1). However, six different studies included in this review described two overlapping patient populations, which was confirmed by the authors[100,119,120,229,236,237]. Therefore, for each separate analysis, the source population was only included once, resulting in 136 separate studies and 627 cases.

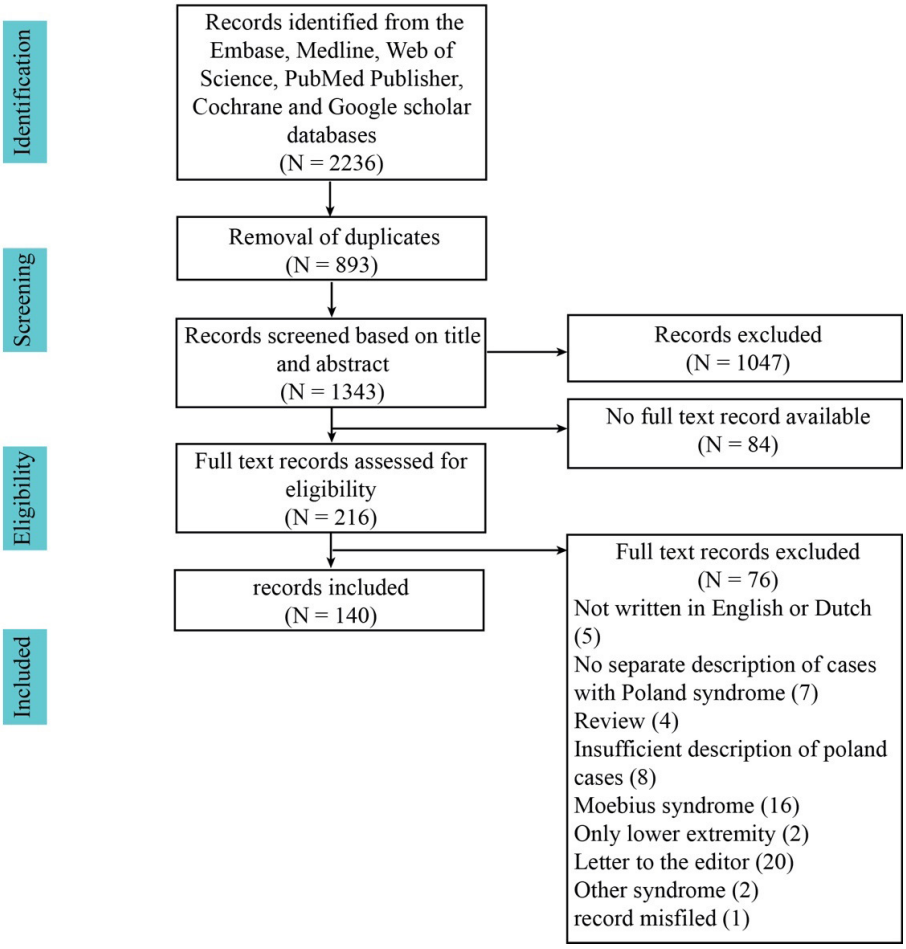

Fig 1: Flow chart illustrates all studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage of the process.

Definition of Poland syndrome

Table 1. The different definitions of Poland syndrome used by the authors of the included articles.

	N	Percentage	References
Total number of studies	**136**	**100%**	
Number of studies with a definition	121	89%	
Definition			
1. Major/Minor pectoral muscle deficiency/aplasia	110	81%	
a With symbrachydactyly (classical definition)	27	20%	3,14,16,17,27,34,52-54,56,57,59,68,73,77,83,87, 88,91,93, 97,114,115,123,129,130,140
b With any upper extremity deformity	30	22%	12,18,20,22,23,29,31-33,35,38,42,47,49,66,75,76, 82,84,86, 98,100,102,105,109,119,131,137,141
c With any hand deformity	19	14%	4,13,15,43,55,62,65,71,72,79,80,90,92 ,110,127,133,139,146
d With optional upper extremity deformity	11	8%	5,39,40,58,111,116,118,122,128 ,135,138
e With syndactyly	11	8%	19,37,41,48,51,63,78,89,113,124,125
f With optional hand deformity	5	4%	10,60,94,106,107
g With optional symbrachydactyly	4	3%	61,70,132,145
h With optional syndactyly	3	2%	46,69,95
2. Only Major/Minor pectoral muscle deficiency/aplasia	9	7%	11,28,44,45,81,99,112,121,126
3. Chest defects	2	1%	30,120

From the 121 studies stating a clear definition of Poland syndrome, we were able to distinguish ten different definitions (Table 1) [99-101,105-114,117-120,122-125,127-130,132-144,146-158,160,161,163-168,170-173,175-190,192-195,197,200-202,204,205,207-211,213-230,232-238,240,241]. Only 27 studies (20%) presented the classical definition of Poland syndrome[99,110-112,122,129,147-149,151,152,154,163,168,172,178,182,184,186,188,192,209,210,218,224,225,235].

Patient characteristics

Out of 627 cases diagnosed with Poland syndrome, data about sex and affected side was available for 618 and 602 patients[99-101,105-118,121-228,230-236,238-241], respectively. Sixty-five percent (n=403) of the patients were male. The right side was affected in 58% (n=352) of the cases, the left side in 39% (n=237), and 2% (n=13) had bilateral deficiencies. Genetic analysis was reported in 24 studies concerning 44 patients[99,105,108,113,115-117,119,120,122,131,143,150,152,168,181,192,198,210,211,216,218,224,230]. In nine of these patients, copy number variations were observed[115,116,120,131,230], such as duplications or deletions of a small region of a chromosome.

Chest and upper extremity deformities in Poland syndrome

Data on chest deformities at the patient level was included for 582 cases (Table 2) [99,100,105-108,110-150,152-225,227,228,230-236,238,239,241]. Hypoplasia or agenesis of the pectoralis major muscle, with or without involvement of the minor pectoral muscle, was described in 577 (99%) cases.

Table 2. The observed thorax deformities in the presented cases derived from the included articles

	N	Percentage
Total number of clinically investigated chest deformities	**582**	**100%**
Description of chest deformity		
Major pectoral muscle agenesis or hypoplasia with optional minor pectoral muscle involvement *	577	99%
Areola or nipple hypoplasia or deformity	246	42%
Breast agenesis or hypoplasia	112	19%
Agenesis or hypoplasia of other muscles	108	19%
Rib deformities	106	18%
Sternal deformities	72	12%
Vertebral deformities	60	10%
Scapula deformities	11	2%
Abnormal diaphragm	3	0.5%

**Patients can have multiple thorax deformities*

Data on hand anomalies at the patient level was included for 588 cases. In 343 cases (58%) an upper extremity deformity was observed (Table 3) [99-101,105-118,121-228,230-236,238-241]. In only 178 cases (29%), did the authors describe a patient with symbrachydactyly, the classic Poland phenotype[99,105,108,110-114,116,117,122,123,127,128,131,132,134,137,139,143,145,146,148,149,152-154,158-162,164-168,170,172,174,175,178-181,184-188,191,193,194,196,198-200,202-209,217-221,223-228,233,234,236,239,241]. In the large cohort studies, Yiyit et al. and Catena et al. reported an incidence of symbrachydactyly of 9% and 40% respectively, in patients diagnosed with Poland syndrome[100,101].

Associated abnormalities

In 277 cases the presence of potentially associated abnormalities was clinically investigated and described in the articles. One or more associated abnormalities were found in 112 cases (cardiovascular (10%), craniofacial (9%), neurological (8%), Sprengel deformity (7%), oncologic (6%), urogenital (4%), respiratory (4%), dermatologic (4%), gastrointestinal (4%), vertebral (3%), hematologic (4%), leukemia (3%), lower extremity (1%), and other abnormalities (3%)) [99,100,105,106,108,111-118,122-125,127,128,130-134,136,138-140,142-146,148,150,152-156,158-164,166,168-173,175-177,180-187,189-196,198,200-202,204,205,208-217,220-222,225,227,228,230-234,236,238,239].

Familial cases

Eleven studies included in our systematic review describe a suggested familial occurrence of Poland syndrome (Table 4) [100,120,121,130,136,181,209,218,221,224,232]. Maternally inherited copy number variants were identified in four patients in the study of Baban et al.[120]. In contrast to the familiar occurrence of Poland syndrome, Stevens et al. describe identical twin sisters without familiar occurrence[224]; one of the sisters had a classic Poland anomaly, the other was unaffected.

Alternative diagnosis

Four different syndromes can present with hand, pectoral, and associated anomalies, namely: Holt-Oram syndrome, Duane-Radial Ray syndrome, Frontonasal dysplasia, and IVIC syndrome[102,103,242,243]. Thirty-eight out of 67 eligible cases had at least 1 feature that matched one of the alternative syndromes[99,108,112,114,115,127,128,131,139,145,152,158,160,161,164,168, 170,172,175,176,181,187,196,198,202,205,208 ,209,220,221,225,227,228,230,233,236]. The alternative diagnosis that matched the presented phenotype most often was Duane-radial ray syndrome (n=32)[99,112,114,115,127,128,131, 139,145,158,160,161, 164,168,170,172,175,176,181,187,196,198,205,208,209,221,225,228,230,236]. The most frequently observed phenotypes that matched one of the syndromes are presented in Table 5.

Table 4. Familial Poland cases

Study	Sex	Familiar relation	Classic Poland anomaly	Thoracic deformities	Upper limb deformity	Other abnormalities
Becker et al 121	M		No	Absent head of the sternal head of the major pectoral muscle and a hypoplastic areola	-	-
	F	Cousin	No	Absent head of the sternal head of the major pectoral muscle, breast hypoplasia and a hypo plastic areola	-	-
	M	Cousin	No	Absent head of the sternal head of the major pectoral muscle and a hypo plastic areola	-	-
Cohen et al. 130	M	-	No	Absent right major pectoral muscle	-	-
	F	Sibling	No	Absent right major pectoral muscle	-	-
Darian et al. 136	F	-	No	Absent major pectoral muscle aplasia of the breast and areolar deformities	-	Ulcerative colitis.
	F	Cousin	No	Absent major pectoral muscle, underdeveloped chest wall and hypoplasia of the breast	-	Ulcerative colitis.
	F	Cousin	No	Absent major pectoral muscle, underdeveloped chest wall and hypoplasia of the breast	-	-
	M	Cousin	No	Absent major pectoral muscle	-	Thoracic teratoma
	M	Cousin	No	Hypo plastic major pectoral muscle	-	-
David et al. 137	M	-	Yes	Absent left sternal head of major pectoral muscle	Ipsilateral brachydactyly and cutaneous syndactyly	-
	M	Grandfather	Yes	Absent left sternal head of major pectoral muscle	Ipsilateral brachydactyly and cutaneous syndactyly	
	M	Cousin	Yes	Absent left sternal head of major pectoral muscle	Ipsilateral brachydactyly and cutaneous syndactyly	
Larrandaburu et al. 181	F	-	Yes	Absent right sternal head of major pectoral muscle and hypoplasia of the breast	Ipsilateral Symbrachydactyly	Psychomotor retardation, bilateral facial palsy, bilateral convergent strabismus, Mobius syndrome

Study	Sex	Familiar relation	Classic Poland anomaly	Thoracic deformities	Upper limb deformity	Other abnormalities
	F	Aunt	No	hypoplasia of right major pectoral muscle and breast	Ipsilateral brachydactyly	Mitral valve prolapse
	F	Cousin	No	-	Triphalangeal thumb	
	F	Cousin	No	-	Triphalangeal thumb	
	M	Cousin	No	-		Cleft palate
	M	Cousin	No	-		Club foot
Rojas-Martínez et al. 209	F	-	No	Hypoplastic major pectoral muscle, breast and areola	Absent hand	Bilateral pes planus
	F	Mother	Yes	Hypoplastic major pectoral muscle, breast and areola	Ipsilateral symbrachydactyly	-
Shalev et al. 218	M	-	No	Hypoplastic right chest	-	-
	F	Mother	No	Hypoplastic right breast, asymmetric areola, fibrocystic changes in left breast	-	-
Soltan et al. 221	F	-	No	Absent sternal head of the major pectoral muscle, amastia and absent areola	-	-
	M	Sibling	No	-	-	Omphalocèle
	F	Cousin	No	-	Transverse hemimelia	-
	M	Cousin	No	-	-	Unilateral microtia
	M	Uncle	No	-	Transverse hemimelia	-
Velez et al. 232	M	-	No	Major pectoral muscle hypoplasia	-	X-linked ichthyosis
	M	Sibling	No	Major pectoral muscle hypoplasia	Simple syndactyly	X-linked ichthyosis
Yiyit et al. 100	Reported a familiar occurrence in 4.4% of the cases (N=113).					
Baban et al. 120	Reported eight cases (4.2%) had at least one relative with a pectoral deformity, sixteen cases (8.4%) had at least one relative with a thoracic or upper limb deformity, but with a normal pectoral muscle.					

Table 5. Observed phenotypes that could fit with one of the alternative syndromes.

Hand anomalies	Other	Associated anomalies		Other
Deficiencies of radio-ulnar axis development		Craniofacial		
Radio-ulnar synostosis	A, B, D		Facial weakness	B
Radial polydactyly	A, B		Ear anomalies	B, C, D
Radial longitudinal deficiency	A, B, D		Hypertelorism	B, C
Radial deficiency of handplate	A, B, D		Broad nasal root	C
Triphalangeal thumb	A, B, D		Epicanthal fold	B, C
Ulnar longitudinal deficiency	B		Bifid nose tip	C
Muscle hypoplasia, other than pectoral muscle	B, D	Eye		
			Strabismus	B, D
			Optic disc anomalies (incl. coloboma)	B, C
		Skeletal		
			Khyphosis/Scoliosis	A, B, D
			Vertebral anomalies	A, B
		Internal organs		
			Renal hypoplasie/agenesis	B
			Atrial septal defect	A, B
			Gastro-intestinal anomalies (incl. pyloris stenos)	B

*Alternative diagnoses: A, Holt-Oram syndrome; B, Duane radial ray syndrome; C, Frontonasal dysplasia, type 1; D, IVIC syndrome.

DISCUSSION

In this systematic review, we studied the phenotypic spectrum of Poland syndrome in the literature. We reviewed 136 articles representing 627 patients, and we compared the applied definitions of Poland syndrome, observed hand, pectoral, and additional anomalies, and familial occurrence. Doing so, we illustrated the broad range of anomalies that were described using the eponym "Poland syndrome". By reviewing atypical cases with multiple congenital anomalies and cross-referencing these cases to syndrome databases (HPO[104], OMIM[97]), we were able to identify that 38 out of 67 atypical cases could fit an alternative diagnosis. Hence, we conclude that the use of the diagnosis Poland syndrome is widely variable in current literature, which can lead to misclassification of a group of patients with pectoral muscle deficiencies. Therefore, we provide a list of phenotypes that might direct to an alternative diagnosis.

The data of the 136 included studies are largely in concordance with the data presented in the three largest studies included in this review[100,101,235]. Patient characteristics such as sex and the distribution of affected sides were comparable with those presented by Yiyit et al. and Catena et al.[100,101]. Furthermore, both Yiyit et al. and Catena et al. present a minority of classic Poland syndrome cases (9% and 40% respectively) in their samples[100,101]. In our systematic review 29% of the cases presented the phenotype described by Alfred Poland[97]. The true proportion of classic cases might be underestimated by both the work presented by Yiyit et al. and our review[100]. Yiyit et al. published multiple articles on atypical Poland syndrome cases and even hypothesized that those cases represent a different syndrome[100,236,237]. Therefore, the number of classic Poland syndrome cases he presents might be an underestimation, as a second syndrome could be highly prevalent in his study sample. Subsequently, our review could present a biased estimation since publication bias might contribute to a higher prevalence of atypical Poland cases in the literature.

Poland syndrome can be considered atypical based on phenotypic presentation as well as familial occurrence. Yiyit et al. described 5 patients with a family history of Poland syndrome and Baban et al. described that, in 24 of the cases, at least one of the features of Poland syndrome was prevalent in the family[100,120]. Unfortunately, not all studies that describe familial Poland cases were available under our library license. Darian summarized several of these non-included studies and showed that 12 studies reported familial Poland cases in which pectoral muscle deficiencies and upper limb anomalies were present [136].

Strikingly, we observed that only 20% of the articles, which referred to the original description by Alfred Poland, used the original phenotype [99,109,111,112,122,129,147-149,151,152,154,163,168,172,178,182,183,186,188,192,209,210,218,224,225,235]. Furthermore, 71% of the patients did not present a hand anomaly that matches the original description of Poland syndrome. Although most of these atypical hand anomalies were etiologically alike, such as brachydactyly and syndactyly, also hand anomalies of completely different etiology were observed, such as cleft hand, radial dysplasia, triphalangeal thumbs and thumb hypoplasia[101,114,128,152,158,165,170,178,181,196,200,235].

Using the correct description of Poland syndrome might seem a semantic dispute; however, the recognition of phenotypic deviation from an established syndrome is a key element in differential diagnosis. This is especially important in Poland syndrome, because the etiology of Poland syndrome is assumed to be multifactorial[120,243], while multiple phenotypically similar syndromes have a genetic cause. Although some of these phenotypically similar syndromes have a genetic cause, only 44 patients in our review received genetic work-up [99,105,108,113,115-117,119,120,122,131,143,150,152,168,181,192,198,210,211,216,218,224,230]. Possibly, for many clinicians and patients the diagnosis of Poland syndrome is sufficiently explanatory for the observed anomalies and therefore no genetic screening is performed. Therefore, an incorrect diagnosis might influence decision making in genetic research.

We identified four possible alterative diagnoses from the HPO dataset[104], syndromes that can also present with pectoral anomalies. For the 67 cases with multiple congenital anomalies, 38 cases presented with at least one of the features of an alternative diagnosis. The most frequently observed overlap was with Duane-Radial-Ray syndrome[102]. The defining phenotypic features of this syndrome are eye anomalies and radial (longitudinal) deficiencies of the upper limb. To illustrate this, the phenotypes presented by Bosch-Banyeras, Parker and Mut could be suggestive for Duane-Radial Ray syndrome [122,191,196]. Likewise, the combination of thumb hypoplasia and cardiac defects (ASD)[114] could be suggestive for Holt-Oram syndrome[103]. However, for both syndromes incomplete penetrance has been described, meaning that cases can present without the associated anomalies. Considering the high prevalence of thumb hypoplasia, the true proportion of patients with Duane-Radial-Ray or Holt-Oram syndrome in our population could easily exceed our estimation. For these unrecognized patients, this might imply that 'hidden' associated anomalies might not be detected due to the lack of diagnostic tests, such as an echocardiogram. Additionally, the recurrence risk for the next generation might be underestimated. However, most importantly, this review only describes those anomalies and syndromes related to pectoral muscle deficiency that were identifiable using a selected search. Chances are that pectoral muscle deficiency also sporadically co-occurs with other syndromes. Especially in the cases with radial deficiencies, we should always rule out more severe syndromes,

such as Fanconi anemia. The odds of misdiagnosing a case with Fanconi syndrome as Poland syndrome might be low, the consequences when the indicated work-up and treatment is not applied are life threatening.

The phenotypic features of the alternative diagnoses we encountered in our study population can be used as a guideline for clinicians encountering atypical Poland syndrome. However, the content of Table 5 is not the complete phenotypic spectrum of these syndromes, but rather an indication of what kind of anomalies could be present. Multiple hand anomalies can be observed with the alternative syndromes. Malformations affecting the radio-ulnar axis of development[5] are suspicious for Holt-Oram syndrome, IVIC syndrome, and Duane-Radial-Ray syndrome[102,103,243]. We also encountered a number of patients with cleft hand and feet [158,235]. Although strictly speaking, there are no relations to pectoralis major deficiencies, there are syndromes which can present with cleft hands and breast aplasia (Ectrodactyly-Ectodermal-Dysplasia or Adult syndrome[244,245]). In general, we therefore consider that any hand anomaly besides symbrachydactyly, should indicate to the clinician the need for a thorough physical examination to detect possible associated anomalies.

In conclusion, there is enough evidence to support our statement that the term Poland syndrome should not be used as a synonym for what, in fact, is pectoral hypoplasia or pectoral deficiency. Pectoral muscle hypoplasia is not distinctive for Poland syndrome alone, but also present in syndromes with other associated anomalies with an entirely different pattern of inheritance. To prevent diagnostic and prognostic errors, in patients with an atypical phenotype we recommend that other syndromes be ruled out before diagnosing a patient with Poland syndrome. As a result, increased attention for patients with Poland-like phenotypes might lead to new evidence concerning the etiology of Poland syndrome or the identification of potential subsyndromes.

APPENDIX A

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	Titlepage (separate from manuscript)
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	1
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	3
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	3-4
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	5
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	5
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	5
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	Appendix 2
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	5
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	5-6
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	5-6

Section/topic	#	Checklist item	Reported on page #
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	NA
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	5-6
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I^2) for each meta-analysis.	5-6
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	NA
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	5-6
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	7, Figure 1
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	7
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	NA
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	NA
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	NA
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	NA
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	NA
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policy makers).	10
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	10

Section/topic	#	Checklist item	Reported on page #
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	10-13
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	Titlepage

From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(7): e1000097. doi:10.1371/journal.pmed1000097

APPENDIX B -SEARCH STRATEGY

Embase.com

('Poland syndrome'/exp OR ('pectoralis major muscle'/exp AND 'hand malformation'/exp) OR ((Poland* NEAR/6 (syndrome* OR syndactyl* OR brachysyndactyl* OR anomal* OR complex* OR symbrachydact* OR sequence* OR deformit* OR ectrosyndact*)) OR ((pectoral* NEAR/3 (muscl* OR dysplas* OR agenes* OR absen*)) AND ((hand NEAR/3 (deformit* OR malformat* OR anomal*)) OR syndact* OR brachysyndactyl*))):ab,ti) AND (phenotype/de OR 'phenotypic variation'/exp OR 'phenotypic plasticity'/exp OR etiology/exp OR pathophysiology/exp OR development/exp OR genetics/exp OR heredity/exp OR 'vascular disease'/exp OR 'blood vessel'/exp OR pathology/exp OR 'familial disease'/exp OR 'disease classification'/exp OR 'disease association'/exp OR (phenotyp* OR pathogen* OR patholog* OR etiopathogen* OR aetiopathogen* OR etiolog* OR aetiolog* OR pathophysiolog* OR characteri* OR heterogen* OR homogen* OR develop* OR genetic* OR inherit* OR heredit* OR famil* OR vascul* OR vessel* OR arter* OR thrombo* OR associat* OR variant* OR variation* OR form OR forms OR bilateral* OR present* OR polymorph* OR caus*):ab,ti)

Medline (OvidSP)

(Poland syndrome/ OR (Pectoralis Muscles/ AND exp Hand Deformities/) OR ((Poland* ADJ6 (syndrome* OR syndactyl* OR brachysyndactyl* OR anomal* OR complex* OR symbrachydact* OR sequence* OR deformit* OR ectrosyndact*)) OR ((pectoral* ADJ3 (muscl* OR dysplas* OR agenes* OR absen*)) AND ((hand ADJ3 (deformit* OR malformat* OR anomal*)) OR syndact* OR brachysyndactyl*))). ab,ti.) AND (exp phenotype/ OR etiology.xs. OR exp causality/ OR pathophysiology.xs. OR exp Growth and Development/ OR "Growth and Development".xs. OR genetics.xs. OR exp genetics/ OR exp heredity/ OR exp vascular diseases/ OR exp blood vessels/ OR pathology.xs. OR exp pathology/ OR exp classification/ OR classification.xs. OR (phenotyp* OR pathogen* OR patholog* OR etiopathogen* OR aetiopathogen* OR etiolog* OR aetiolog* OR pathophysiolog* OR characteri* OR heterogen* OR homogen* OR develop* OR genetic* OR inherit* OR heredit* OR famil* OR vascul* OR vessel* OR arter* OR thrombo* OR associat* OR variant* OR variation* OR form OR forms OR bilateral* OR present* OR polymorph* OR caus*).ab,ti.)

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Google scholar

"Poland|Polands syndrome|anomaly|complex|sequence|deformity" phenotype|pathogenesis|pathology|etiopathogenesis|etiology|aetiology|pathophysiology|characterization|development|genetic|genetics|vascular|vessel|thrombosis|variant|variation|forms|causality

Human Phenotype Ontology database

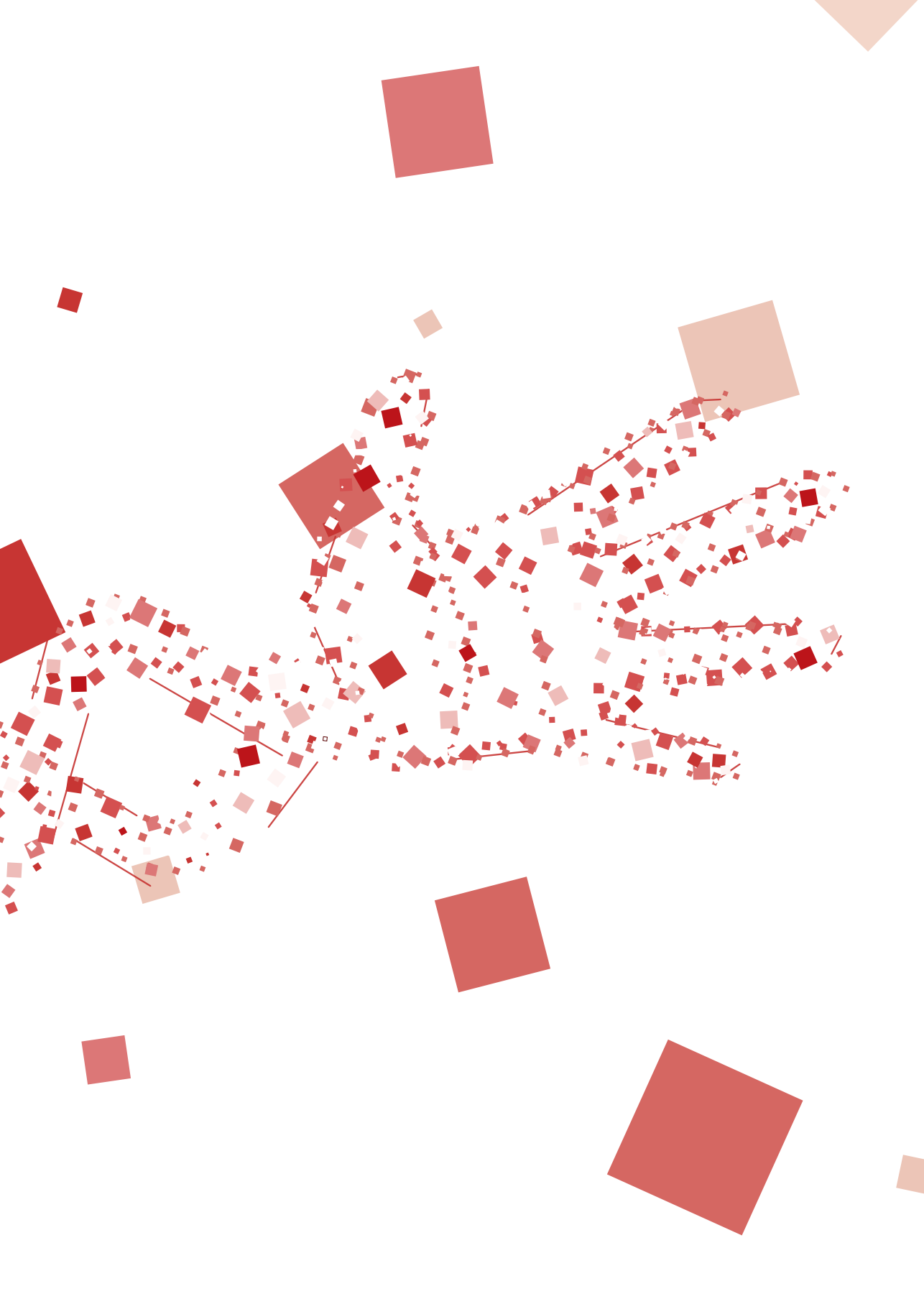
File name: ALL_SOURCES_ALL_FREQUENCIES_diseases_to_genes_to_phenotypes.txt
Accession date: February 22th of 2017

Used phenotypic terms for pectoral muscle deficiency:

Pectoral muscle hypoplasia/aplasia	HP:0005258
Pectoralis major hypoplasia	HP:0008953
Pectoralis hypoplasia	HP:0008998
Aplasia of the pectoralis major muscle	HP:0009751

PART 2

Evaluation of the Classification Strategy in Population Studies



5

Intrafamilial Variability of the Triphalangeal Thumb Phenotype in a Dutch Population: Evidence for Phenotypic Progression over Generations?

Martijn Baas; Jacob W.P Potuijt; Steven E.R. Hovius; A. Jeannette M. Hoogeboom; Robert-Jan H. Galjaard; Christianne A. van Nieuwenhoven

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ABSTRACT

Triphalangeal thumbs (TPT) are regularly caused by mutations in the ZRS in LMBR1. Phenotypic variability can be present in TPT-families. However, recent observations suggest an increased occurrence of severe phenotypes in the Dutch TPT-population. Therefore, the aim of this study is to investigate the progression of the clinical severity of TPT-phenotype through generations.

Index patients from a Dutch TPT-population were identified. A 105C>G mutation in the ZRS has previously been confirmed in this population. Questionnaires regarding family occurrence and phenotypes were distributed. Subsequently, families were visited to validate the phenotype. Both occurrence and inheritance patterns of the TPT-phenotype were analyzed through multiple generations.

170 patients with TPT were identified from 11 families. When considering all 132 segregations (parent-to-child transmission), 54% of the segregations produced a stable phenotype, 38% produced a more severe phenotype while only 8% of the phenotype was less severe when compared to the affected parents. Overall, 71% of the index patients had a more severe phenotype compared to their great-grandparent.

Although all family members share an identical mutation in the ZRS (105C>G), it does not explain the wide phenotypic range of anomalies. Our observational study provides better estimations for counseling and provides new insights in the long-range regulation of SHH by the ZRS-enhancer.

In the current study, we provide evidence that the assumed variability in TPT-phenotype is not random, but in fact it is more likely that the expression becomes more severe in the next generation. Therefore, we observe a pattern that resembles phenotypic anticipation in TPT-families.

INTRODUCTION

The triphalangeal thumb (TPT) is a congenital upper limb anomaly in which the thumb has three phalanges. TPT can present as a single isolated malformation (e.g. preaxial polydactyly type II; OMIM:174500), or can be part of a complex hand anomaly accompanied with additional polydactyly and syndactyly.[246,247] Furthermore, triphalangeal thumbs can also be seen in numerous syndromes, such as Holt-Oram syndrome or Duane-Radial Ray syndrome.[248]

In the southern part of the Netherlands, a PPD2 population with an estimated prevalence of 1:1000 has previously been described and studied.[249] These reports already have shown a broad phenotypic variation in PPD2. Multiple studies were performed to elucidate the genetic cause of TPT in these Dutch families. In 1994, Heutink et al. located the locus for TPT to 7q36 (LOD: 12,61).[250] In 2002, Lettice finally identified the 105C>G mutation in the ZPA-Regulatory Sequence (ZRS), that resides in intron 5 of the LMBR1 gene. The mutation in the ZRS was confirmed in all affected members in a sample of 200 patients from aforementioned southern population in the Netherlands.[250,251]

In the first phenotypic description of this population in 1994, most reviewed patients had a triphalangeal thumb, either with or without an additional preaxial ray. However, already a number of cases in the initially described population had a complex phenotype[249], including multiple preaxial rays, postaxial duplications and/or syndactyly of digits 3-5. The intra- and inter-familial variability of the phenotype among patients with the same genotype that has been observed in the Dutch TPT-families, has also been described in other families in literature and was accepted to be a natural variation of the phenotype.[247,251-253]

Recent observations in our clinic, however, suggest an increased familial occurrence of complex hand anomalies in successive generations of TPT families, which could indicate phenotypic progression through generations instead of intra- and inter-familial variability of the phenotype. This alternative hypothesis is supported by family history, which commonly revealed that the affected parent or grandparent of these more complex affected children had a less severe TPT phenotype. This observation is illustrated in Figure 1, by showing the phenotypes of 3 patients from subsequent generations within one family.

In order to determine whether recent observations of more complex phenotypes in newborns are incidental events or a structural pattern of phenotypic anticipation with progressive complexity among multiple families, the Dutch TPT population was revisited. The aim of this study is to investigate the progression of the severity of the TPT phenotype through generations. Secondarily, we aim to provide better estimates for phenotypic differences within families to determine the risk of a less severe, stable and more severe phenotypes for future children.

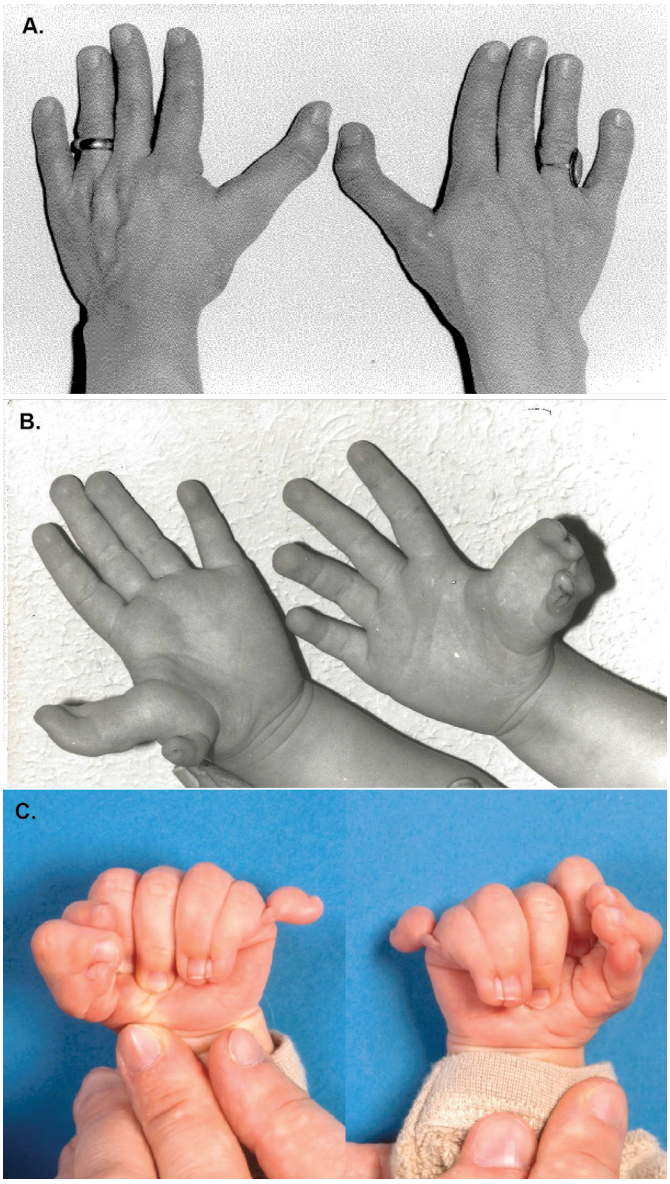

Figure 1. *TPT-phenotype of 3 subsequent generations from the TPT-population.* ***A:*** *Post-operative image of grandmother of index-patient. During the operation, an additional thumb on both hands was removed.* ***B:*** *Pre-operative image of mother of index-patient, presenting with a triplication of the thumb on the left hand and a quadruplication of the thumb on the right hand.* ***C:*** *Pre-operative image of the index patient. The index patient has a symmetrical phenotype on both hands; with a triplication of the thumb, syndactyly between digits 4 and 5. All patients were born with a postaxial polydactyly, but were removed prior to the photographs in grandmother and mother of index patient.*

METHODS

Index patients were identified from the database of patients with a congenital upper limb anomaly in the Sophia Children's Hospital who visited the clinic between 1972 and 2014. All medical records of patients with either a registered triphalangeal thumb or preaxial polydactyly were reviewed for normal preoperative photo's as well as X-rays. Patients with at least one preaxial ray or with a triphalangeal component, with or without family history at referral, were eligible for inclusion. Eligible patients were contacted and subsequently a questionnaire was distributed among the index patients or their parents. The aim of the questionnaire was to create a pedigree by identifying other affected family members of the participant.

It is important that all families that were included in this study, have an identical mutation in the ZRS, as various mutations in the ZRS causes different TPT-phenotypes. For example, 295C>T mutations provide a mild phenotype[254], whereas mutations at position 404 of the ZRS cause a severe phenotype.[247,255,256] To ensure a genetic homogenous population, only patients whom ancestors originate from the small region in the South-West of the Netherlands (in which the 105C>G mutation is highly prevalent) were included. Genetic homogeneity has previously been established in families in this population.[247,250] In order to confirm the genetic homogeneity in this population, genealogical research was performed by reviewing the municipal archives to identify a common ancestor that connects all individual families with each other.

Additionally, local general practitioner's records were reviewed to identify patients that were not referred to the Sophia Children's Hospital. If affected family members were operated on in other hospitals, consent to acquire their medical records was requested. Furthermore, families were visited to confirm the phenotype of the affected family members (using family/birth photos) and to gather additional familial information. Relatedness of the family members was investigated through genealogical research. Sub-pedigrees were established using only family members with confirmed TPT phenotypes. Although multiple sub-pedigrees were produced, all family members originated from one larger pedigree with one common ancestor. In order to analyze the phenotypic pattern of TPT among patients, the phenotype of every patient in the different sub-pedigrees was categorized among 6 types in consecutive order of complexity of phenotype (Figure 2). Patients have phenotype Type I when only an isolated TPT is present. Type VI encompasses an extensive phenotype, with both additional preaxial and postaxial malformations combined with another aberration, for example polydactyly of the feet. The intermediate types of TPT were categorized based on the hypotheses of increasing levels of ectopic SHH-signaling in the anterior margin of the limb bud and subsequently disruption of SHH-signaling in the posterior margin of the limb bud.

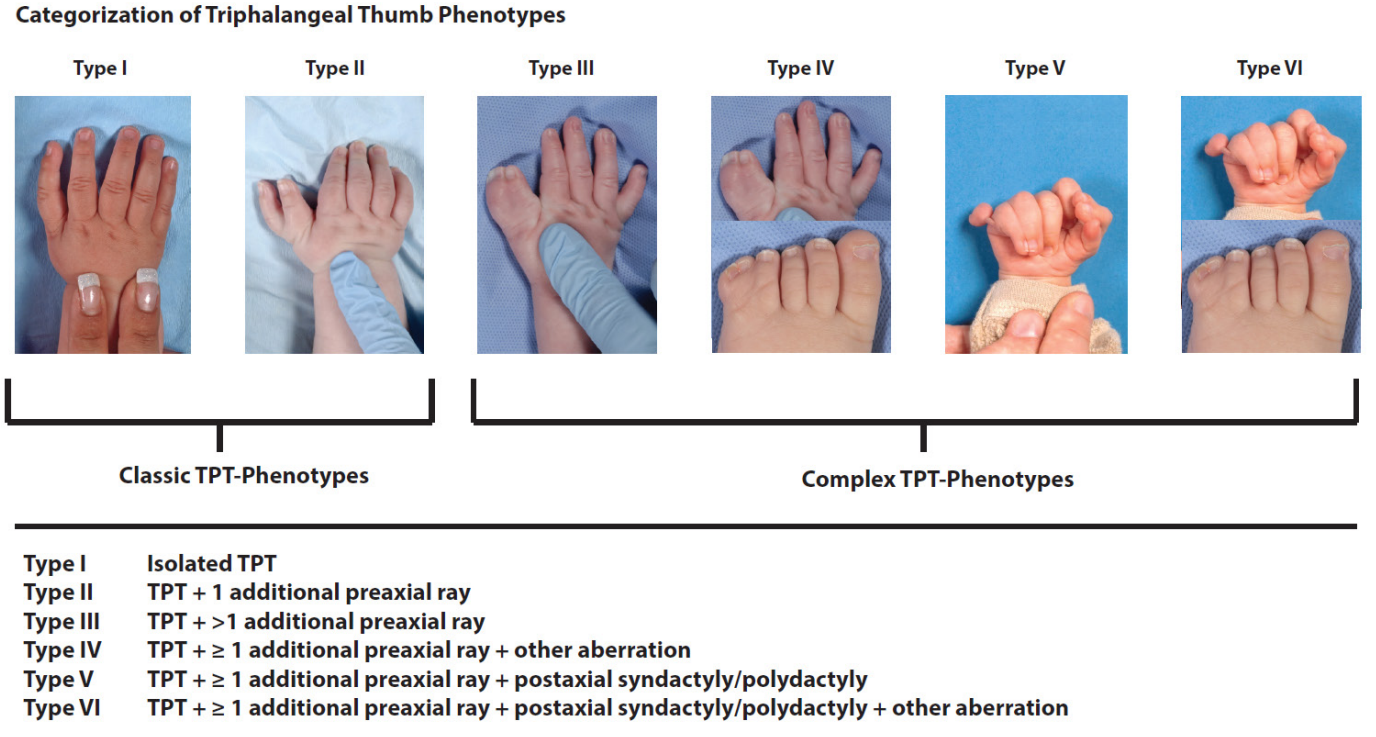

Figure 2. *Categorization of TPT-phenotypes according to complexity. Type I and II are considered a classic phenotype. Type 3 or higher have additional aberrations and therefore are categorized as a complex TPT-phenotype.*

According to the initial prevalence of phenotypes in the investigated population[249], type I (isolated TPT) and II (TPT with preaxial polydactyly) were considered 'classic' phenotypes (preaxial polydactyly type II, OMIM:174500). Phenotypes type III and higher were regarded as complex TPT phenotypes.

Patients have been arranged in 3 birth cohorts: from 1890 to 1940, from 1940 to 1990 and from 1990 to 2015. These cohorts were selected in order to compare affected patients of different generations with each other. Family members of the first and second generation were born before 1940, whereas patients of the third and fourth generation were born between 1940 and 1990. The youngest cohort consisted of affected family members who were born after 1990.

After all phenotypes in the sub-pedigrees were established, the variation of the phenotype over the different generations in the family was obtained. The transmission of the phenotype was regarded 'stable' when the phenotype is the same in the analyzed ancestor (parent, grandparent and great-grandparent) and child. When a child has a more complex phenotype than their affected ancestor, the transmission is considered 'more severe'. In the opposite case, when a less complex phenotype of TPT is observed in the child in comparison with their parent, the inheritance pattern is described as a 'less severe' transmission of the phenotype.

The transmission of the TPT phenotypes was obtained for all individual segregations as well as the sum of consecutive transmissions (up to 5 generations). Analyzing phenotypical inheritance through multiple generations may display a more distinct inheritance pattern of TPT-phenotype in these families and might provide a better estimation of the risk of developing a more complex phenotype in subsequent offspring for clinical consultation.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 21. Proportions were tested using a Chi-square test, unless specified differently. Variability was defined as an equal chance of non-stable phenotypes being more severe or less severe as compared to the ancestor, therefore to statistically test deviation from this theory the H_0 hypothesis of 50% contribution (of either subgroup of non-stable phenotypes) was assumed for the Student's T-test.

Ethics

Our research proposal (MEC-2015-278) has been approved by the Medical Ethics Committee of the Erasmus University Medical Centre in Rotterdam, The Netherlands.

RESULTS

In total, 46 index patients from the southern part of the Netherlands were suitable for inclusion, 8 patients were not included due to expired contact information. Furthermore, 3 patients were not willing to participate in this study. Therefore, 35 index patients were included in the study from 11 branches of the family. Genealogical research confirmed relatedness of the 11 branches of the family by identifying a common ancestor who was born in 1731. All index patients showed triphalangealism of at least one preaxial ray on the preoperative X-ray. Through questionnaires and provided information of the index patients, we identified 135 additional family members with TPT. In total, 170 patients were included in this study. The 105C>G mutation in the ZRS was confirmed in multiple different branches in this pedigree (Figure 3).

Table 1. Overview of included pedigrees

Pedigree Number	Number of Lineages (N)	Patients with TPT (N)	Generations per Lineage	More Severe	Less Severe
1	13	38	4,23 (3,00 - 5,00)	8	0
2	9	31	4,44 (3,00 - 5,00)	7	0
3	6	25	3,33 (2,00 - 4,00)	5	0
4	4	19	4,25 (3,00 - 5,00)	1	2
5	4	11	3,50 (3,00 - 4,00)	2	0
6	3	10	3,00	1	0
7	3	10	3,66 (3,00 - 4,00)	3	0
8	3	8	3,33 (3,00 - 4,00)	1	0
9	1	7	4,00	1	0
10	1	6	4,00	0	0
11	1	5	3,00	0	0
Total	**48**	**170**	**3,90**	**29**	**2**

Table 1. demonstrates an overview of characteristics of the included sub-pedigrees. Each pedigree is formed by of a certain number of lineages. Lineages consist of subsequent affected family members of different generations. A lineage of 3 generations contains 3 subsequent patients with TPT: a child, a parent and a grandparent. The average number of relatives contributing to a lineage was 3,90. Therefore, the average analysis of the inheritance pattern is between child and their great-grandparent. The number of patients with a TPT within these sub-pedigrees varies from 5 to 38 patients.

In total, the TPT phenotype segregated 132 times. Segregation is defined as a transmission of the TPT-phenotype from a parent to child. As we want to analyze the pattern of segregation in these sub-pedigrees, only segregations were included in the analysis when the phenotype of both the parent and child were known.

The distribution of classic and complex TPT phenotypes is displayed over the 3 different birth-cohorts (from 1890 to 1940, from 1940 to 1990 and from 1990 to 2015) in Figure 4. The observed percentage of complex phenotypes among these groups was 6%, 21% and 54% respectively. The distribution between the groups was significantly different ($p<0,001$).

If the phenotype of the youngest generation is compared to their oldest identified ancestor, the phenotype was more severe in 29 patients, the phenotype remained stable in 17 patients while the phenotype became less severe in only 2 patients, as illustrated in Table 1. Assuming the presence of intrafamilial variability as null hypothesis (the probability of a more severe phenotype equals the probability of a less severe phenotype), the differences found are significantly different ($p<0,001$, Student's t-test, $H_0=0.5$).

Considering, all 132 segregations of the phenotype, we observed that 54% of the segregations produced a stable phenotype in the next generation, whereas in 38%, the phenotype was more severe and in 8% the phenotype was less severe when compared to the affected parent. In the cases in which the phenotype was not stable, the chance of a more complex phenotype is 5.6 times higher than the chance of a less severe phenotypes. When the analysis is expanded to two and three subsequent segregations we observed that the subsequent segregations produce a more severe phenotype in respectively 54% and 71% of the observed cases (Figure 5).

The result of segregation, if the phenotype of the parent was taken into account, is displayed in Figure 6a-c. Given that the parent has an isolated TPT (6a), the child had a more severe phenotype in 50% of cases. Most of these children had an additional preaxial polydactyly. However, when the parent already had an additional preaxial polydactyly (6b), the children had a complex phenotype in 28% of the cases, like additional triplications and/or postaxial (syn)polydactyly. Moreover, once the parent already had a complex phenotype, all children had complex phenotypes as well. No regression to either isolated TPT or TPT with preaxial polydactyly was observed within this offspring.

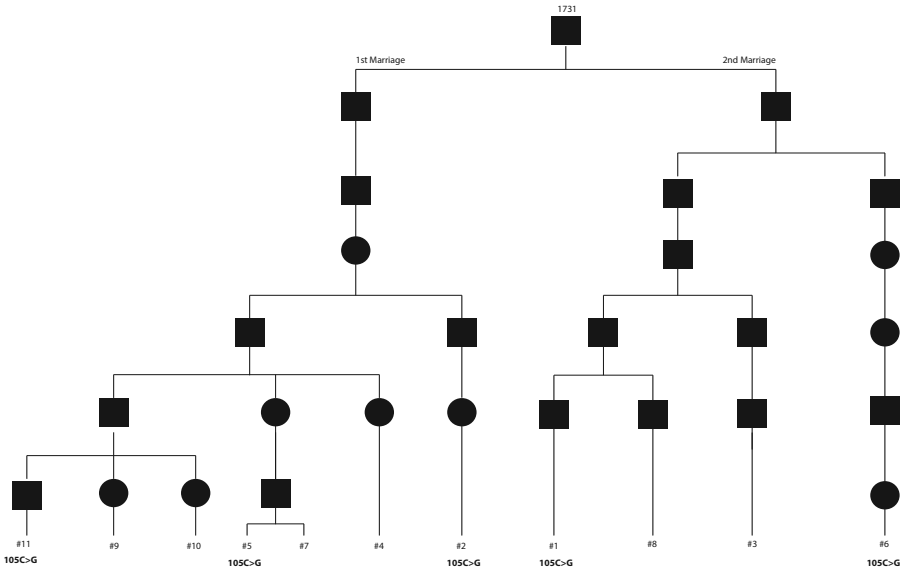

Figure 3. *Pedigree of the included 105C>G TPT-population. Analyzed families are presented with their family number at the bottom of the pedigree. A 105C>G mark below the family number indicates that a 105C>G mutation in the ZRS-region was confirmed within this family. A common ancestor of all families was identified as a man who was born in 1731.*

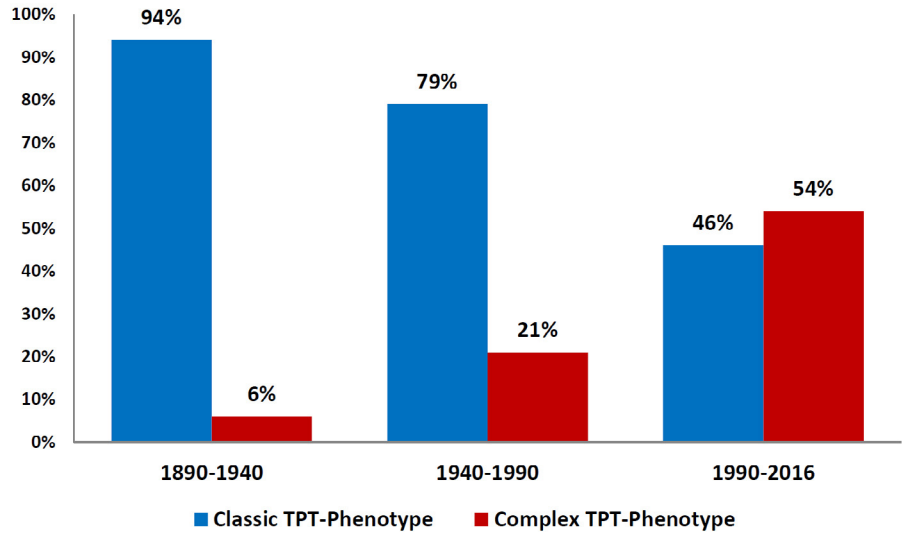

Figure 4. *Distribution of phenotypes among 3 birth cohorts. A significant increase of complex TPT phenotypes is observed when comparing the cohort '1990 - 2016' with the other cohorts (chi-square<0,001).*

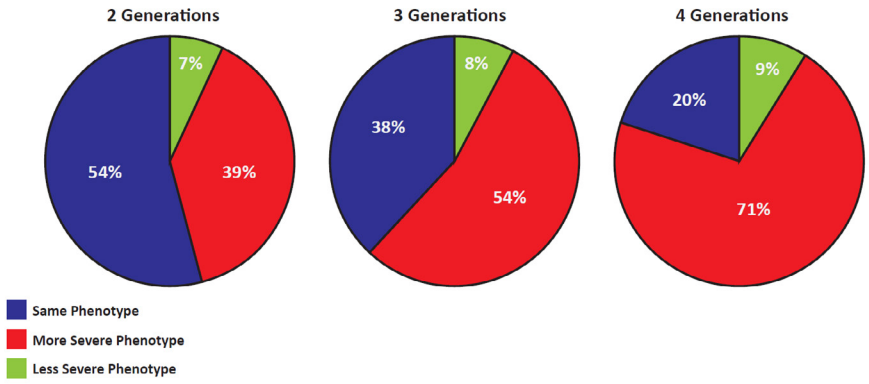

Figure 5. *Distribution of phenotypic transmission in TPT families when taking multiple consecutive generations into account. (2 consecutive generations; n=132, 3 consecutive generations; n=90, 4 consecutive generations; n=45)*

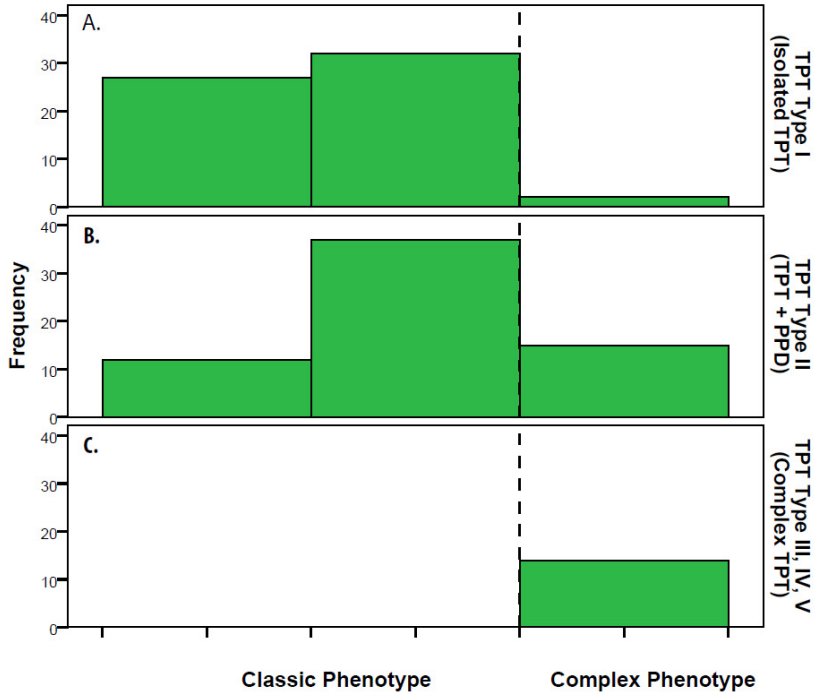

Figure 6. *Distribution of offspring phenotype based on parental phenotype.* ***A:*** *Distribution of the child's phenotype if the parent had an isolated TPT.* ***B:*** *Distribution of the child's phenotype if the parent had TPT with an additional thumb.* ***C:*** *Distribution of the child's phenotype if the parent had a complex phenotype.*

DISCUSSION

In this study, we illustrate that the severity of an inherited phenotype of affected patients in triphalangeal thumb families is not random, but rather shows a pattern of increased severity over subsequent generations. We found that in 39 percent of the cases, the phenotype increased in severity in one generation whereas only 7% decreased in severity. As a result, we found that in consecutive generations the phenotype of the youngest patients was more severe that the phenotype of their ancestor in 71% of the evaluated segregation lines. The probability of progression depends on the phenotype of the parent and varies from 28% to 50% in classic TPT-phenotypes. If the parent presents with a complex phenotype (type III – type VII), no regression to a milder phenotype can be expected. Counselors can use this information to prepare future parents for the possible increase in severity of the phenotype in TPT-families.

Our observations are based on the Dutch TPT-population previously described by Zguricas et al., interestingly the same mutation in the ZRS found in this Dutch TPT family has been described in a Chinese Han Family. Although this Chinese family shared the 105C>G mutation [257], they predominantly have isolated triphalangeal thumbs and there was no indication for progression of the phenotypic among this four-generation family. The results presented in this study must therefore be interpreted with care as the inclination towards complex phenotypes in the Dutch population might not be generalizable to all TPT populations and could be induced by factors that are specific to the Dutch TPT-population.

By retrospectively studying a formally isolated TPT-population, bias might be introduced affecting the validity and generalizability of the presented results. First, the validity might be affected due to recall bias in the obtained phenotypes, especially in the cohort born between 1890 and 1940. Therefore, we repeated analysis in the sub-pedigrees, disregarding the oldest birth cohort (thus including only those patients born between 1940 and 2015) and found similar phenotypic progression rates that correspond with the primary reported analysis. Second, inbreeding has been reported in other studies that have investigated this population, which could affect generalizability. We reviewed the presence of inbreeding in our cohort and found that inbreeding was predominantly present in the founding population (birth cohort 1750 to 1850). Additionally, our pedigree data did not reveal the presence of inbreeding in the included TPT-families. Third, the generalizability of increasing severity of the phenotype over generations can be questioned due to the isolated population and their rare 105 C>G mutation. However, phenotypic progression over generations can also be observed in several other families that have been reported in literature.[254,258,259] Considering the repeated analysis when disregarding the oldest cohort, the lack of evidence

for inbreeding and the presence of other families in which the phenotype seems to progress, we conclude that although these biases might be present, they are unlikely to contribute much to our observations.

To be able to hypothesize on the pathophysiology of our findings, we must understand the molecular mechanism of the ZRS in relation to the observed phenotypes. If the ZRS is properly folded and bound with transcription factors that are spatiotemporally unique to the Zone of Polarizing Activity (ZPA) the ZRS is functionally active and upregulates SH expression to specify digit number and morphology. The specification of digit number and identity has been predominantly attributed to a morphogenic SHH-gradient across the limb bud, hypothesized by Wolpert's morphogen gradient model.[260] Higher concentrations and longer duration of SHH-expression at the posterior side of the limb bud result in the development of digits IV and V. Absence of SHH-concentration in the anterior side of the limb bud will conversely create a bi-phalangeal thumb. Point mutations in the ZRS disrupt normal SHH-patterning in the limb and cause ectopic SHH-expression on the anterior margin of the limb bud, which can result in a triphalangeal thumb.[255] Advancing on the SHH-gradient model, triplications and quadruplications of the thumb will presumably be caused by increasing concentrations of ectopic SHH expression in the anterior limb bud. Furthermore, loss of SHH function in the ZPA causes postaxial polydactyly in animal models.[261] The observed postaxial polydactyly in the Dutch TPT-population could therefore be caused by reduced SHH expression in the posterior limb bud. Besides the SHH-gradient model, an analogy can be made to the pathophysiology of Greig Cephalopolysyndactyly syndrome. Patients with Greig syndrome commonly present with combined pre-axial, post-axial polydactyly and syndactyly which is caused by GLI3 mutations. SHH is an important mediator for GLI3 to active GLI3A transition in the limb bud. Point mutations in the ZRS therefore might influence the same pathway as GLI3 mutations in the posterior margin of the limb bud.[262]

The progression of the phenotype could be explained by the many differences the Dutch population might have with the Chinese 105 C>G population. We hypothesize two different causes for the observed phenotypic progression. First, phenotypic progression might be attributable to genetic or molecular factors that influence SHH-expression in the ZPA in the limb bud additional to the 105 C>G mutation. Secondly, generation specific environmental or parental factors could be present and might interfere with normal limb development through epigenetic modulation of the genome.

The first genetic factor could be somatic mosaicism. Increasing polydactyly phenotypes have been described as a result of somatic mosaicism of a point mutation in the ZRS.[263] Subsequently, somatic mosaicism could explain a more

severe phenotype of the child than the phenotype of the parent. However, somatic mosaicism fails to explain phenotypic anticipation in multiple subsequent generations.

An alternative genetic hypothesis is that the progressive phenotypes in TPT-families are due to a second genetic locus introduced by the primarily non-affected parent which modifies the effect of the original 105C>G mutation. Although the presence of an additional influencing locus in an isolated population is a valid hypothesis, the extent of observations among different segregation lines in different sub-pedigrees and an example of two half siblings sharing the same affected father with both the same increasing phenotype all devaluate this hypothesis.

The last genetic hypothesis is based upon the fact that the observed phenotypic progression could be regarded as phenotypic anticipation. Phenotypic anticipation is a well-described phenomenon in repeat disorders such as Huntington disease, which is genetically caused by increasing CAG repeat sequences in the *HTT* gene. Although phenotypic anticipation is not known within the field of congenital upper limb malformations, repeat sequence disorders have been described in both *HOXD13* and *HOXA13* related synpolydactyly phenotypes.[264-266] However, synpolydactyly phenotypes in HOXD13 and HOXA13 repeat sequence disorders show limited intrafamilial variability with an exception for consanguineous families, in which patients with homozygous mutations do show far more severe hand anomalies.[267] Many subsequent research groups evaluated the TPT genotype in the past, resulting in the identification of various point mutations in the ZRS. Although repeat sequences were not found in the linkage analysis by Heutink et al., exon-trapping analysis performed in later studies[268] of the candidate region would have identified exonic repeats in the REPEATMASKER analysis. The presence of intronic repeats outside the ZRS, however, cannot be excluded and should be further investigated.

There are several arguments that could support environmental or parental specific factors as a cause for phenotypic progression in TPT-families. First of all, generation specific environmental factors, such as the stimulated use of folic acid during pregnancy from the early 90's onwards, could have explained the higher occurrence of severe phenotypes in the youngest birth cohort, however fails to explain the earlier progression as observed in the pedigree in Figure 7. In this pedigree, both classic and complex phenotypes have been observed in the same generation. It is therefore unlikely that such generation specific factors are causative to the changing phenotypes.

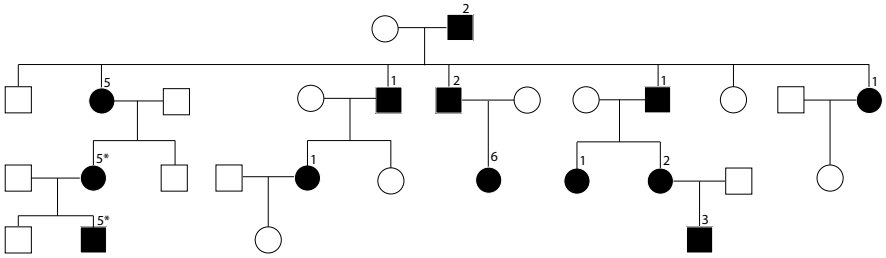

Figure 7. *Section of the pedigree of Family 1. Various types of transmissions (stable, severe and less severe) are observed in the different segregation lines. Patients with TPT phenotype 5* had a triplication or quadruplication of the thumb and were therefore considered more severely affected than a TPT phenotype 5.*

A second environmental hypothesis might be the increasing paternal age. Also, the large changes in family planning over the last century could lead to a change in parental age at conception. As Zhu et al. have suggested, high paternal age might increase the overall incidence of congenital malformations.[269] We performed a preliminary analysis on parental age in a limited number of segregation lines, but did not find enough support for the influence of the age of the parents.

The last environmental hypothesis is the significant increase of obesity in the western population over the past 25 years. Parental obesity has been widely associated with a higher risk of birth defects in newborns. The notion that parental obesity might also play a role in phenotypic progression of the TPT phenotype cannot be disregarded.[270]

This study underlines the importance of thorough phenotypical assessment in studies on familial disease. In order to explore the causality of the structural evidence of phenotypic progression in these TPT-families, additional molecular genetic research and revisiting the Dutch population is required. Expression assays in transgenic animal models still remain the standard in molecular genetic research on limb development. We encourage future collaborations between clinicians, geneticists and developmental biologists that will lead to a more comprehensive understanding of the role of SHH and its regulatory elements on congenital limb anomalies like triphalangeal thumb and polydactyly.

6

Preaxial polydactyly of the foot: Clinical and genetic implications for the orthopedic practice based on a literature review and 76 patients

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ABSTRACT

Preaxial polydactyly of the foot is a rare malformation and clinicians are often unfamiliar with the associated malformations and syndromes. In order to give guidelines for diagnostics and referral to a clinical geneticist, we provide an overview of the presentation using literature review and our own patient population.

Literature review was based on the Human Phenotype Ontology (HPO) project. From the HPO dataset, all phenotypes describing preaxial polydactyly were obtained and related diseases were selected. An overview was generated in a heatmap, in which the phenotypic contribution of 12 anatomical groups to each disease is displayed. Clinical cases were obtained from our hospital database and were reviewed in terms of phenotype, genotype, heredity, and diagnosed syndromes.

From the HPO dataset, 21 diseases were related to preaxial polydactyly of the foot. The anatomical groups with the highest phenotypic contribution were lower limb, upper limb, and craniofacial. From our clinical database, we included 76 patients with 9 different diseases, of which 27 had a *GLI3* mutation. Lower limb malformations (n=55), upper limb malformations (n=59), and craniofacial malformations (n=32) were most frequently observed. Malformations in other anatomical groups were observed in 27 patients.

Preaxial polydactyly of the foot often presents with other upper and lower limb malformations. In patients with isolated preaxial polydactyly of the foot, referral to a clinical geneticist is not mandatory. In patients with additional malformations, consultation of a clinical geneticist is recommended. When additional limb malformations are present, analysis of *GLI3* is most feasible.

INTRODUCTION

Polydactyly of the foot is a congenital malformation which can be classified as preaxial: extra hallux; postaxial (most common): extra fifth toe; and central (rarest): middle 3 toes involved. Despite the high prevalence of hand and foot polydactyly in newborns, preaxial polydactyly of the foot is rare. A recent report on congenital limb defects in the northern part of the Netherlands estimated a birth prevalence of 0.4 patients per 10,000 births in preaxial polydactyly of the foot compared to 2.1 patients per 10,000 births in preaxial polydactyly of the hand.[3]

Although rare, knowledge about the presentation of preaxial polydactyly of the foot is important, because almost half of the cases multiple congenital anomalies, such as syndactyly and atrial septum defects, have been reported.[3] Furthermore, preaxial polydactyly of the foot may be associated with syndromes including more severe malformations, such as craniosynostosis or corpus callosum agenesis.[271,272] Due to the low prevalence, most clinicians are unfamiliar with these additional malformations and associated syndromes, which may therefore not be recognized. The surgical literature mainly addresses the surgical treatment of preaxial polydactyly [273-275], whereas the geneticists mainly focus on the genetic background of polydactyly, such as studies on *GLI3* and *HOXD13.* [276,277] The lack of a clear overview of phenotypes that present with preaxial polydactyly of the foot makes it difficult for the surgeon to identify associated malformations and to recognize related syndromes. Associated malformations may be minimal or their detection requires additional diagnostic methods, such as an echocardiogram for cardiovascular anomalies. Therefore, a clear overview of the phenotypic and genotypic characteristics would be helpful.

To clarify the phenotypic and genotypic characteristics of syndromes and diseases which can present with preaxial polydactyly of the foot, we combined review of genetic databases with clinical evaluation of a large surgical population with preaxial polydactyly of the foot. The combination of information from genetic databases and a case series will lead to a more complete overview of the malformation, together with a practical guideline for referral to the clinical geneticist.

METHODS

Review of the human phenotype ontology (HPO) database

We extracted all diseases which can present with preaxial polydactyly of the foot from the HPO dataset.[104] Data extraction was performed according to the CulaPhen protocol [25], which was modified to select only phenotypes related to preaxial polydactyly of the foot. The protocol uses the HPO annotation files accessible at the HPO's Jenkins page. Accession date, search terms used for this extraction and the URL are available in Appendix 1. A wide spectrum of HPO terms were used (from "broad hallux" to "mirror image polydactyly") to ensure inclusion of all possible diseases. Both subclasses and parental classes were included to assure that all related diseases were included. All diseases that were obtained through this search were manually reviewed by MB and EB to confirm the presence of preaxial polydactyly in the phenotypic descriptions of that disease in literature. For each of the diseases that passed manual review, a list of standardized phenotypes according to HPO nomenclature was available. These HPO phenotypes were categorized based on the Rotterdam registration form for congenital upper anomalies and the CulaPhen protocol (12 groups: CULA, Circulatory, Respiratory, Digestive, Urogenital, Nervous System, Vertebral Column, Musculoskeletal, Head/Neck, Lower limb, Skin, Others).[8,25] For each disease, the number of phenotypes among the 12 different anatomical groups was counted and was expressed in a ratio reflecting the contribution of that anatomical group to the total disease presentation. The obtained ratios can be converted to a heatmap in which the contribution of that anatomical group to the total disease presentation is expressed by a color gradient (0=white, 1=red). If multiple subtypes of a disease were present, the individual diseases were grouped. In addition, when possible the diseases in the heatmap were grouped according to the classification of genetic skeletal disorders.

Review of clinical patients

Our hospital database was retrospectively searched for patients with preaxial polydactyly of the foot diagnosed between 1993 and 2016. All subjects were reviewed in terms of phenotype, sex, heredity, and present gene mutations and syndromes. Assessment of phenotypes in these patients was done based on review of documentation on clinical examination performed by the clinical geneticist and other specialized clinicians. Also, documentation of medical imaging and blood tests were used to identify internal congenital malformations. Because children repeatedly visit the hospital for follow-up of their foot problems until the age of 18, additional verification of malformations presenting at a later

age was also performed using medical documentation. Congenital malformations were classified in 12 different anatomical groups, similar to the groups used in the classification of phenotypes in the genetic databases.

At first consultation at our department, a clinical geneticist decided if genetic testing was indicated. Genetic testing usually consisted of array analysis and targeted sequencing of candidate genes (such as *GLI3*, *FGFR2*, etc.). Alternatively, if a first degree relative with the same congenital condition was already diagnosed with a genetic disease, this diagnosis was considered valid for the included patient as well. Patients without gene mutations documented in the patient documentation were classified as test not indicated, results not present in patient documentation, or no mutation found in genetic testing.

Ethics, funding, and potential conflicts of interest

The institutional medical ethics committee (MEC) reviewed the protocol and agreed that MEC approval was not needed for this study (MEC-2015-679), November 10, 2015. The project was funded by the Esser Foundation. No competing interests were declared.

RESULTS

Review of the HPO database

We selected 13 HPO phenotypes that could match preaxial polydactyly of the foot from the HPO database (Appendix 1). Using these phenotypes, we extracted 123 different diseases. By manual literature review, we excluded 83 diseases. The remaining 40 diseases included 9 diseases with multiple subtypes. Combining the different subtypes in 1 disease group led to a total of 21 unique diseases. The related genes to these diseases are presented in Appendix 2. Most of these diseases (18/21) can be grouped in 3 main categories: polydactyly/syndactyly/triphalangeal syndromes, syndromes with craniofacial malformations (including craniosynostosis), and syndromes with mental retardation as a key aspect [6]. Out of the 3 remaining diseases, 2 are ciliopathies and 1 is a dysplasia syndrome [6]. The anatomical groups that contributed the most to the 21 diseases were lower limb, upper limb, craniofacial, and nervous system. Disease specific contributions of the anatomical groups are presented in Figure 1. The phenotypic presentation of preaxial polydactyly of the foot and examples of the related phenotypes are presented in Figure 2.

Database Rotterdam

Preaxial foot polydactyly was present in 76 patients (Table 1). 55 patients were bilaterally affected. Most cases (n=41) were hereditary. In 3 patients, familial occurrence could not be confirmed due to adoption (n=2) or donor conception (n=1).

Nine out of 21 disease entities and syndromes reported in the HPO dataset were present in our population (Table 2). Besides syndrome diagnosis, 3 different subtypes of preaxial polydactyly (PPD) were used in clinic: type 1, 2 and 4.[13] 9 cases showed PPD type 1, characterized by only preaxial polydactyly of the feet and/or the hands. 3 cases showed PPD type 2, characterized by preaxial polydactyly of the feet and triphalangeal thumbs or halluces. 8 cases showed PPD type 4, characterized by 'crossed polydactyly' (preaxial polydactyly of the feet with postaxial polydactyly of the hands). Preaxial polydactyly of the foot was often accompanied with hand, foot, and craniofacial malformations. 27 patients were affected with malformations in other anatomic groups (Table 2). Twenty-two patients never received a genetic test or test results were not documented. In 5 of the 6 patients with unilateral PPD type 1, genetic testing was never performed. In contrast, all patients with a triphalangeal thumb and preaxial polydactyly (PPD type 2) were tested for genetic mutations.

Table 1. Patient characteristics of the observed population with preaxial polydactyly of the foot.

Characteristics	N = 76
Sex	
Male	30
Female	46
Affected foot	
Right	15
Left	6
Bilateral	55
Hereditary	
Yes	41
No	32
Unknown	3

In the cohort that was tested for genetic mutations, genetic testing was performed in 39 patients and in 15 affected parents of the patients. In 43 cases this resulted in confirmation of a mutation (Table 3). A *GLI3* mutation was confirmed in the largest part of the population (n=27). In patients with only hand and foot malformations, 14 out of 16 confirmed mutations were in *GLI3*. In patients with anomalies in the different anatomical groups, 13 out of 27 confirmed mutations were in *GLI3*.

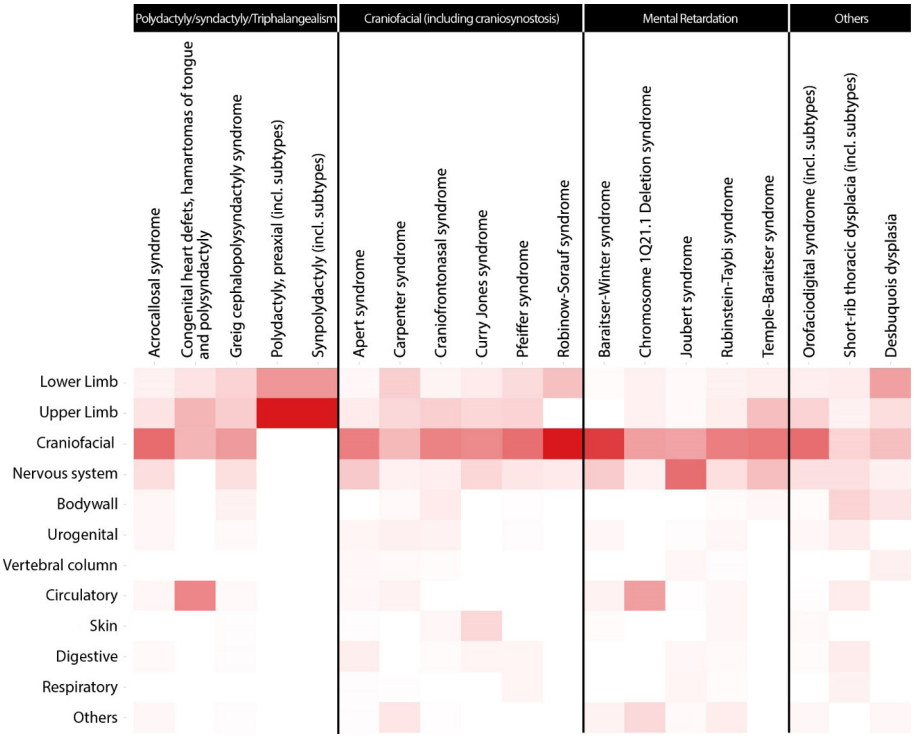

Figure 1. Heatmap showing the contribution of each anatomical group per disease related to preaxial polydactyly of the foot.

The contribution of each anatomical group per disease is expressed by a red color gradient. No contribution = white; maximal contribution = red. The group of preaxial polydactyly consists of preaxial polydactyly type 1, preaxial polydactyly type 2, and preaxial polydactyly type 4. These subtypes are considered as independent disease entities, but are combined in one column because contribution of each anatomical group is similar in every type.

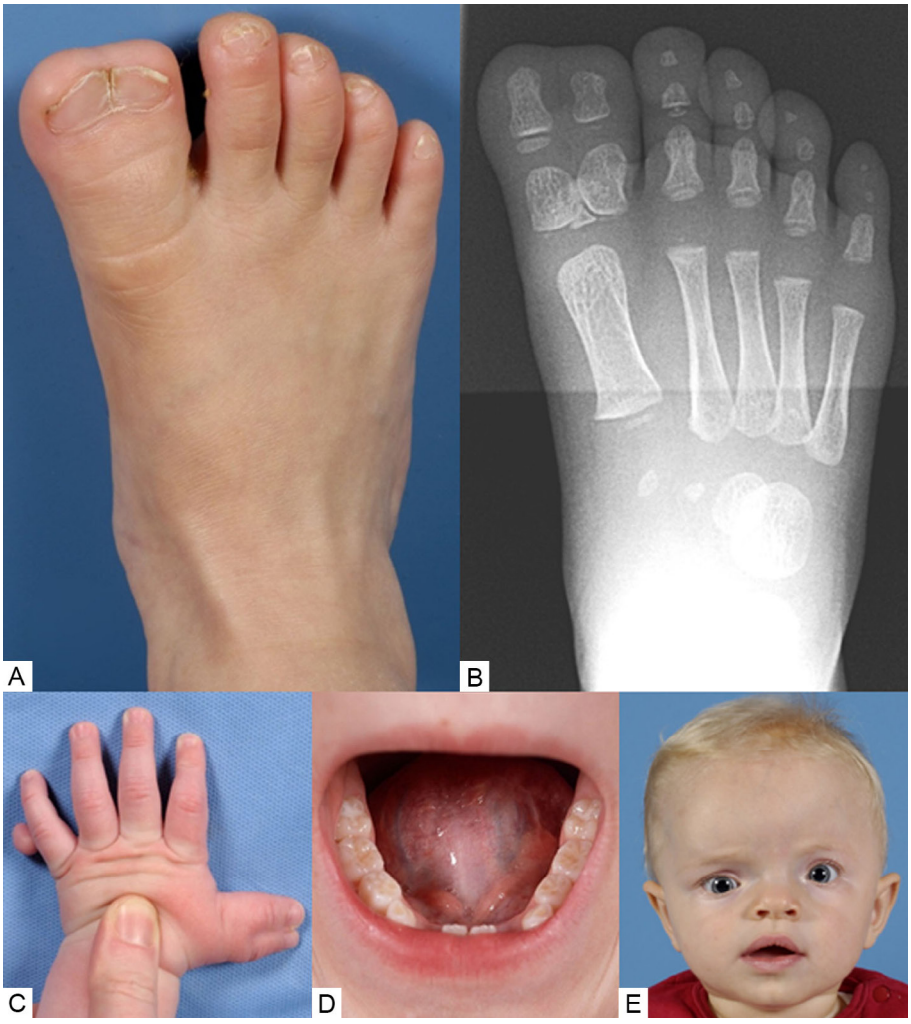

Figure 2. Example of preaxial polydactyly of the foot and related phenotypes.
A) Clinical photograph of preaxial polydactyly of the foot. B) X-ray of preaxial polydactyly of the foot. C) Typical hand malformation in Greig syndrome: Preaxial and postaxial polydactyly of the hand. D) Typical malformation in orofacial-digital syndrome: Tongue malformation. E) Typical craniofacial malformations in craniofacial dysplasia syndrome: Craniosynostosis, hypertelorism, and asymmetric face.

Table 2. Phenotypes of the specific syndromes and diseases in the observed population

	Syndrome	Acrocallosal syndrome	GLI3-mediated-polydactyly	Preaxial polydactyly type I	Preaxial polydactyly type II	Preaxial polydactyly type IV	Apert syndrome	Carpenter syndrome	Cranio-frontonasal dysplasia	Pfeiffer syndrome	Saethre-Chotzen syndrome	Oro-facial-digital syndrome	Multiple malformations, no disease diagnosis	Total N
	Total N	**1**	**26**	**9**	**3**	**8**	**7**	**2**	**2**	**1**	**2**	**2**	**13**	**76**
1	**Lower limb***	1	25	1	2	7	7	2	1	1	0	1	7	**55**
2	**Upper limb**	1	24	1	3	8	7	2	0	1	2	1	9	**59**
3	**Craniofacial**	1	11	0	0	0	7	2	2	1	2	2	4	**32**
4	**Neurological**	1	1	0	0	0	0	0	0	0	0	0	1	**3**
5	**Body wall**	1	0	0	0	0	0	0	1	0	1	1	3	**7**
6	**Urogenital**	0	0	0	0	0	0	2	0	1	0	0	1	**4**
7	**Vertebral**	0	0	0	0	0	0	0	0	0	0	0	0	**0**
8	**Circulatory**	0	1	0	0	0	2	2	0	1	0	1	4	**11**
9	**Dermatological**	0	1	0	0	0	0	0	0	0	1	0	3	**5**
10	**Digestive**	0	0	0	0	0	0	1	0	0	1	0	0	**2**
11	**Respiratory**	0	1	0	0	0	0	1	0	1	0	0	0	**3**
12	**Other**	0	1	0	0	0	5	0	1	1	1	0	2	**11**

*Other lower limb malformations than preaxial polydactyly of the foot.

Table 3. Genetic testing and observed gene mutations

Genes	Total (N = 76)	Cases with exclusively upper/lower limb malformations (N=35)	Cases with other anomalies besides upper/lower limb malformations (N=41)
EFBN1	2	0	2
FGFR2	8	0	8
GLI3	27	14	13
LMBR1	1	1	0
RAB23	2	0	2
TBX5	1	1	0
TWIST1	2	0	2
Genetic test not performed	15	10	5
No mutation found	11	6	5
No test result documented	7	3	4

DISCUSSION

Evaluation of the genetic databases showed that 21 disease entities are associated with preaxial polydactyly of the foot. However, the spectrum of observed malformations and disease entities in our own population only included 9 disease entities. Our series mainly consisted of *GLI3*-mediated polydactyly, PPD type 1, and PPD type 4. This observation shows that patients with preaxial polydactyly of the foot commonly present without malformations in other anatomic groups. Therefore, the combination of genetic databases and patient populations in rare malformations or diseases is needed to create a thoroughly, but also realistic picture for clinical practice.

When focusing on the phenotypic presentation of preaxial polydactyly of the foot, 3 main groups in our patient population can be distinguished. The first group includes patients with an isolated preaxial polydactyly without any other anomalies. The second group includes patients with combined hand and foot malformations, but without severe anomalies in other parts of the body. The third group includes patients with preaxial polydactyly of the foot and several anomalies in other parts of the body.

The first group, patients with an isolated preaxial polydactyly of the foot, are not commonly tested for genetic mutations in our clinic: most patients with a unilateral preaxial polydactyly in our population were never tested for genetic mutations. Reason for limited testing in isolated preaxial polydactyly is the low detection rate of mutations in patients with isolated limb anomalies.[66] Furthermore, Orioli and Castilla (1999) showed that most cases of isolated preaxial polydactyly of the foot occur sporadically.[278] However, in a molecular review by Johnston et al. (2005) 2 patients from a GLI3 family presented with bilateral isolated preaxial polydactyly of the foot.[279] Conclusively, genetic testing might be justified for bilateral and/or familial cases. Nevertheless, in most cases with isolated preaxial polydactyly of the foot testing for a mutation has little consequences for clinical practice.

The second distinctive group is formed by patients with additional limb malformations. Often occurring limb malformations in patients with preaxial polydactyly of the foot are preaxial and postaxial polydactyly of hands and feet, in combination with syndactyly, also named PPD type 4. These patients with multiple limb malformations are often successfully tested for *GLI3* mutations. When specific craniofacial features, such as frontal bossing, macrocephaly, hypertelorism, and a broad nasal bridge, are also present, this phenotype can be classified as Greig syndrome.[280] However, craniofacial malformations in patients with Greig syndrome can be minimal and easily missed, which makes the distinction between PPD type 4 and Greig syndrome difficult.[281] Therefore, in our population we have chosen

to classify patients with a *GLI3* mutation as *GLI3*-mediated polydactyly in order to avoid bias due to the retrospective character of this study and underreporting of craniofacial anomalies in our patient documentation.

The third group of patients with preaxial polydactyly of the foot is clinically distinctive by several malformations in different organ systems besides preaxial polydactyly of the foot. Specific features of these patients, such as craniosynostosis or cardiac septal defect, lead to a differential diagnosis resulting in a focused search for gene mutations and eventually syndrome diagnosis. Despite the focused search for gene mutations, a mutation cannot be found in all patients. This is illustrated in our population by the group of patients with multiple congenital anomalies, but without a disease diagnosis (n=13). The combination of malformations found in these patients could be coincidental. However, it is also possible that these patients suffer from a disease that was not recognized in counseling, or they might have a different genetic mutation not addressed in targeted analyses. In the end, based on our population study we would advise that at least any patient with several malformations in different organ systems should be referred to a clinical geneticist for evaluation.

Although our study provides an overview of the phenotypic and genotypic spectrum of patients with preaxial polydactyly of the foot, it cannot be used for any measure of risk or prevalence in this population because there is no birth registration for limb malformations in the southern part of the Netherlands. In addition, our distribution of included phenotypes could be influenced by selection bias. However, both isolated preaxial polydactyly of the foot and more complex phenotypes are present in our patient population, which makes selection bias based on patients' phenotypes less likely. Furthermore, the retrospective character might have led to underreporting of specific features due to absence of a standardized research protocol for clinical examination prior to the introduction of the Rotterdam registration form for congenital anomalies.[8] Nevertheless, previous literature reported that one third of patients with preaxial polydactyly of the foot do have a recognized condition, which is comparable in our patient population. Lastly, the actual prevalence of genetic aberrations might be underestimated. Genetic testing in our population consisted of targeted tests of commonly affected genes. Next Generation Sequencing (NGS) would allow for all related genes to be tested at once, which might improve the diagnostic yield due to the detection of variants in the less commonly affected genes.

We distinguished the different phenotypes associated with preaxial polydactyly of the foot from both literature and our clinical experience. Our research is a starting point in the search for suspected syndromes presenting with preaxial polydactyly of the foot. Furthermore, we formulated a practical guideline for referral to a clinical geneticist. In patients with isolated preaxial polydactyly of the foot, referral to a clinical geneticist is not mandatory. Detection rate of gene mutations is low in these patients and implications for clinical practice in case of genetic mutations are limited. When additional limb malformations are present besides preaxial polydactyly of the foot, *GLI3* mutations are likely and consultation of a clinical geneticist should be considered to discuss genetic testing. In patients with multiple malformations in different parts of the body, referral to a clinical geneticist is advised to obtain a complete phenotypic description of the malformations, followed by specified genetic testing in order to confirm or exclude syndrome diagnosis.

APPENDIX 1.

HPO data source and HPO codes for all phenotypes related to preaxial polydactyly of the foot

Search terms HPO database	HPO - code
Preaxial polydactyly	HP:0100258
Preaxial foot polydactyly	HP:0001841
Foot polydactyly	HP:0001829
Polysyndactyly of hallux	HP:0005873
Duplication of the phalanx of the hallux	HP:0010066
Duplication of the proximal phalanx of the hallux	HP:0010093
Partial duplication of the distal phalanx of the hallux	HP:0010097
Broad hallux	HP:0010055
Broad phalanx of the toes	HP:0010174
Broad hallux phalanx	HP:0010059
Broad distal hallux	HP:0008111
Broad distal phalanx of the hallux	HP:0010077
Mirror image polydactyly	HP:0010689

Data source: http://compbio.charite.de/jenkins/job/hpo.annotations.monthly/
Dataset: ALL_SOURCES_ALL_FREQUENCIES_diseases_to_genes_to_phenotypes.txt
Last accession date: 22/02/2017

APPENDIX 2.

Genes related to the selected diseases

Disease	OMIM/Orphanet ID	Related Gene
Acrocallosal Syndrome	OMIM:200990	KIF7
Apert Syndrome	OMIM:101200	FGFR2
Baraitser-Winter Syndrome	OMIM:243310	ACTB
Carpenter Syndrome (incl subtypes)	OMIM:201000, ORPHANET:65759	MEGF8, RAB23
Chromosome 1Q21.1 Deletion Syndrome, 1.35-Mb	OMIM:612474	GJA5, GJA8
Congenital Heart Defects, Hamartomas Of Tongue, And Polysyndactyly; CHDHTP	OMIM:217085	WDPCP
Craniofrontonasal Syndrome	OMIM:304110	EFNB1
Curry Jones Syndrome	ORPHANET:1553	SMO
Desbuquois Dysplasia	OMIM:251450	CANT1
Greig Cephalopolysyndactyly Syndrome	OMIM:175700	GLI3
Joubert Syndrome (incl. subtypes)	ORPHANET:475, ORPHANET:220493, ORPHANET:2318	AHI1, ARL13B, B9D1,C5ORF42, CC2D2A, CEP104, CEP290, CEP41, CSPP1, HYLS1, INPP5E, KIAA0556, KIAA0586, MKS1, TCTN1, TCTN2, TMEM138, TMEM216, TMEM231, TMEM237, TMEM67, ZNF423
Orofaciodigital Syndrome (incl. subtypes)	OMIM:277170, ORPHANET:2750, OMIM:258860	C5ORF42, OFD1, TCTN3
Pfeiffer Syndrome (incl. subtypes)	OMIM:101600, ORPHANET:93258, ORPHANET:93259, ORPHANET 93260	FGFR1, FGFR2
Polydactyly, Preaxial (incl. subtypes)	OMIM:174500	LMBR1 (ZRS, Intron 5)
Robinow-Sorauf Syndrome	OMIM:180750	TWIST1
Rubinstein-Taybi Syndrome (incl. subtypes)	OMIM:180849, OMIM:613684	CREBBP, EP300
Short-Rib Thoracic Dysplasia (incl. subtypes)	OMIM:263520	NEK1
Synpolydactyly (incl. subtypes)	OMIM:186000	HOXD13
Temple-Baraitser Syndrome	OMIM:611816	KCNH1

PART 3

Genotype-Phenotype Correlation

7

Variant type and position predict two distinct limb phenotypes in patients with GLI3-mediated polydactyly syndromes

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ABSTRACT

Pathogenic DNA variants in the *GLI3* gene are known to cause multiple syndromes: e.g. Greig syndrome, Preaxial Polydactyly-type 4 (PPD4) and Pallister-Hall syndrome. Out of these, Pallister-Hall is a different entity, but the distinction between Greig syndrome and PPD4 is less evident. Using latent class analysis (LCA), our study aimed to investigate the correlation between reported limb anomalies and the reported *GLI3* variants in these GLI3-mediated polydactyly syndromes. We identified two sub-classes of limb anomalies that relate to the underlying variant.

Both local and published cases were included for analysis. The presence of individual limb phenotypes was dichotomized and an exploratory LCA was performed. Distribution of phenotypes and genotypes over the classes were explored and subsequently the key predictors of latent class membership were correlated to the different clustered genotypes.

297 cases were identified with 127 different variants in the *GLI3* gene. A two-class model was fitted revealing two subgroups of patients with anterior versus postaxial anomalies. Postaxial anomalies were observed in cases with truncating variants in the activator domain (postaxial polydactyly; hand OR: 12,7; foot OR: 33,9). Multivariate analysis supports these results (Beta: 1,467, p=0,013 and Beta: 2,548, $p<0.001$, respectively). Corpus callosum agenesis was significantly correlated to these variants (OR: 8.8, $p<0.001$).

There are two distinct phenotypes within the GLI3-mediated polydactyly population: preaxial and postaxial orientated. Variants that likely produce haploinsufficiency are associated with preaxial phenotypes. Postaxial phenotypes are associated with truncating variants in the activator domain. Patients with these truncating variants have a greater risk for corpus callosum anomalies.

INTRODUCTION

GLI-Kruppel family member 3 (*GLI3*) encodes for a zinc finger transcription factor which plays a key role in the sonic hedgehog (SHH) signaling pathway essential in both limb and craniofacial development.[45,282] In hand development, SHH is expressed in the zone of polarizing activity (ZPA) on the posterior side of the handplate. The ZPA expresses SHH, creating a gradient of SHH from the posterior to the anterior side of the handplate. In the presence of SHH, full length GLI3-protein is produced (GLI3A), whereas absence of SHH causes cleavage of GLI3 into its repressor form (GLI3R).[43,283] Abnormal expression of this SHH /GLI3R gradient can cause both pre- and postaxial polydactyly.[2]

Concordantly, pathogenic DNA variants in the *GLI3* gene are known to cause multiple syndromes with craniofacial and limb involvement, such as: Acro-collosal syndrome[272] (OMIM:200990), Greig cephalopolysyndactyly syndrome[284] (OMIM:175700), and Pallister-Hall syndrome[285] (OMIM:146510). Also, in non-syndromic polydactyly, such as preaxial polydactyly-type 4 (PPD4, OMIM:174700)[286], pathogenic variants in *GLI3* have been described. Out of these diseases, Pallister-Hall syndrome is the most distinct entity, defined by the presence of central polydactyly and hypothalamic hamartoma.[287] The other *GLI3* syndromes are defined by the presence of preaxial and/or postaxial polydactyly of the hand and feet with or without syndactyly (Greig syndrome, PPD4). Also, various mild craniofacial features such as hypertelorism and macrocephaly can occur. Pallister-Hall syndrome is caused by truncating variants in the middle third of the *GLI3* gene.[46,279,288] The truncation of GLI3 causes an overexpression of GLI3R, which is believed to be the key difference between Pallister-Hall and the *GLI3*-mediated polydactyly syndromes.[279,287] Although multiple attempts have been made, the clinical and genetic distinction between the GLI3-mediated polydactyly syndromes is less evident. This has for example led to the introduction of sub-Greig and the formulation of an Oro-facial-digital overlap syndrome.[46] Other authors, suggested that we should not regard these diseases as separate entities, but as a spectrum of *GLI3*-mediated polydactyly syndromes.[289]

Although phenotype/genotype correlation of the different syndromes has been cumbersome, clinical and animal studies do provide evidence that distinct regions within the gene, could be related to the individual anomalies contributing to these syndromes. First, case studies show isolated preaxial polydactyly is caused by both truncating and non-truncating variants throughout the *GLI3* gene, whereas in isolated postaxial polydactyly cases truncating variants at the C-terminal side of the gene are observed.[288,290] These results suggest two different groups of variants for pre- and post-axial polydactyly. Secondly, recent animal studies suggest that postaxial malformations in GLI3-mediated polydactyly syndromes are likely related to a dosage effect to GLI3R rather than due to the influence of an altered GLI3A expression.[291]

Past attempts for phenotype/genotype correlation in GLI3-mediated polydactyly syndromes have directly related the diagnosed syndrome to the observed genotype.[46,279,288,292] Focusing on individual hand phenotypes, such as pre- and post-axial polydactyly and syndactyly might be more reliable because it prevents misclassification due to inconsistent use of syndrome definition. Subsequently, latent class analysis (LCA) provides the possibility to relate a group of observed variables to a set of latent, or unmeasured, parameters and thereby identifying different subgroups in the obtained dataset.[293] As a result, LCA allows us to group different phenotypes within the GLI3-mediated polydactyly syndromes and relate the most important predictors of the grouped phenotypes to the observed *GLI3* variants.

The aim of our study was to further investigate the correlation of the individual phenotypes to the genotypes observed in GLI3-mediated polydactyly syndromes, using LCA. Cases were obtained by both literature review and the inclusion of local clinical cases. Subsequently, we identified two sub classes of limb anomalies that relate to the underlying *GLI3* variant. We provide evidence for two different phenotypic and genotypic groups with predominantly preaxial and postaxial hand and feet anomalies, and we specify those cases with a higher risk for corpus callosum anomalies.

METHODS

Literature review

The Human Gene Mutation Database (HGMD Professional 2019) was reviewed to identify known pathogenic variants in *GLI3* and corresponding phenotypes.[294] All references were obtained and cases were included when they were diagnosed with either Greig or sub-Greig syndrome or PPD type 4.[46,279,288] Pallister-Hall syndrome and Acrocollasal syndrome were excluded because both are regarded distinct syndromes and rather defined by the presence of the non-hand anomalies, than the presence of pre- or postaxial polydactyly.[289,295] Isolated pre- or postaxial polydactyly were excluded for 2 reasons: the phenotype/genotype correlations is better understood and both anomalies can occur sporadically which could introduce falsely assumed pathogenic *GLI3* variants in the analysis. Additionally, cases were excluded when case-specific phenotypic or genotypic information was not reported or if these two could not be related to each other. Families with a combined phenotypic description, not reducible to individual family members, were included as one case in the analysis.

Clinical cases

The Sophia Children's hospital database was reviewed for cases with a *GLI3* variant. Within this population, the same inclusion criteria for the phenotype were valid. Relatives of the index patients were also contacted for participation in this study, when they showed comparable hand, foot, or craniofacial malformations or when a *GLI3* variant was identified. Phenotypes of the hand, foot, and craniofacial anomalies of the patients treated in the Sophia Children's hospital were collected using patient documentation. Family members were identified and if possible, clinically verified. Alternatively, family members were contacted to verify their phenotypes. If no verification was possible, cases were excluded. The research protocol was approved by the local ethics board of the Erasmus MC University Medical Center (MEC 2015-679).

Phenotypes

The phenotypes of both literature cases and local cases were extracted in a similar fashion. The most frequently reported limb and craniofacial phenotypes were dichotomized. The dichotomized hand and foot phenotypes were preaxial polydactyly, postaxial polydactyly, and syndactyly. Broad halluces or thumbs were commonly reported by authors and were dichotomized as a presentation of preaxial polydactyly. The extracted dichotomized craniofacial phenotypes were

hypertelorism, macrocephaly, and corpus callosum agenesis. All other phenotypes were registered, but not dichotomized.

Pathogenic GLI3 Variants

All *GLI3* variants were extracted and checked using Alamut Visual 2.14. If indicated, variants were renamed according to standard Human Genome Variation Society nomenclature.[296] Variants were grouped in either missense, frameshift, nonsense or splice site variants. In the group of frameshift variants, a subgroup with possible splice site effect were identified for sub-group analysis when indicated. Similarly, nonsense variants prone for nonsense mediated decay (NMD) and nonsense variants with experimentally confirmed NMD were identified.[297] Deletions of multiple exons copy number variations and translocations were excluded for analysis. A full list of included mutations is available in the supplementary materials.

The location of the variant was compared to 5 known structural domains of the *GLI3* gene: 1) repressor domain, 2) zinc finger domain, 3) cleavage site, 4) activator domain, which we defined as a concatenation of the separately identified transactivation zones, the CPB binding domain and the mediator binding domain (MBD) and 5) the MID1 interaction region domain.[44,282,284,298,299] The boundaries of each of the domains were based on available literature (Figure 1, exact locations available in the supplementary materials). The boundaries used by different authors did vary, therefore a consensus was made.

Latent class analysis (LCA)

To cluster phenotypes and relate those to the genotypes of the patients, an explorative analysis was done using LCA in R (R version 3.6.1 for Mac; Polytomous variable LCA, poLCA version 1.4.1). We used our LCA to detect the number of phenotypic subgroups in the dataset and subsequently predict a class membership for each case in the dataset based on the posterior probabilities.

In order to make a reliable prediction, only phenotypes that were sufficiently reported and/or ruled out were feasible for LCA, limiting the analysis to preaxial polydactyly, postaxial polydactyly, and syndactyly of the hands and feet. Only full cases were included. To determine the optimal number of classes, we fitted a series of models ranging from a one-class to a six-class model. The optimal number of classes was based on the Akaike Information Criterion (AIC), the Bayesian Information Criterion (BIC) and the obtained entropy.[300] The explorative LCA produces both posterior probabilities per case for both classes and predicted class membership. Using the predicted class membership, the phenotypic features per class were determined in

a univariate analysis (Chi-Square, SPSS version 25). Using the posterior probabilities on latent class (LC) membership, a scatter plot was created using the location of the variant on the x-axis and the probability of class membership on the y-axis for each of the types of variants (Tibco Spotfire, version 7.14) . Using these scatter plots, variants that give similar phenotypes were clustered.

Genotype/phenotype correlation

Because a latent class has no clinical value, the correlation between genotypes and phenotypes was investigated using the predictor phenotypes and the clustered phenotypes. First, those phenotypes that contribute most to LC-membership were identified. Second those phenotypes were directly related to the different types of variants (missense, nonsense, frameshift, splice site) and their clustered locations. Quantification of the relation was performed using a univariate analysis using a Chi-Square test. Because of our selection criteria, meaning patients at least have two phenotypes, a multivariate using a logistic regression analysis was used to detect the most significant predictors in the overall phenotype (SPSS version 25). Finally, we explored the relation of the clustered genotypes to the presence of corpus callosum agenesis, a rare malformation in GLI3-mediated polydactyly syndromes which cannot be readily diagnosed without additional imaging.

RESULTS

We included 251 patients from the literature and 46 local patients[46,66,279,288,292,297,301-317], in total 297 patients from 155 different families with 127 different *GLI3* variants. Thirty-two of which were large deletions, copy number variations or translocations. In 6 local cases, the exact variant could not be retrieved by status research.

The distribution of the most frequently observed phenotypes and variants are presented in Table 1. Other recurring phenotypes included developmental delay (n=22), broad nasal root (n=23), frontal bossing or prominent forehead (n=16), and craniosynostosis (n=13), Camptodactyly (n=8) and a broad first interdigital webspace of the foot (n=6).

Table 1. Baseline phenotypes and genotypes of selected population

Phenotypes		**Affected/reported cases (n)**
Hand	Preaxial polydactyly	124/294
	Postaxial polydactyly	170/292
	Syndactyly	124/297
Foot	Preaxial polydactyly	238/297
	Postaxial polydactyly	70/295
	Syndactyly	193/297
Cranium	Macrocephaly	85/228
	Hypertelorism	92/237
	Corpus callosum	16/145
Genotypes		**Cases (n)**
Included in analysis	Frameshift	107
	Nonsense	68
	Missense	60
	Splice	24
Excluded in analysis	CNV	29
	Translocation	3
	No specific information on mutation	6

The LCA model was fitted using the 6 defined hand/foot phenotypes. Model fit indices for the LCA are displayed in Table 2. Based on the BIC, a 2-class model has the best fit for our data. The 4-class model does show a gain in entropy, however with a higher BIC and loss of degrees of freedom. Therefore, based on the majority of performance statistics and the interpretability of the model, a 2-class model was chosen. Table 3 displays the distribution of phenotypes and genotypes over the 2 classes.

Table 1 Depicts the baseline phenotypes and genotypes in the obtained population. Note incomplete data especially in the cranium phenotypes. In total 259 valid genotypes were present. In total, 289 cases had complete data for all hand and foot phenotypes (preaxial polydactyly, postaxial polydactyly and syndactyly) and thus were available for LCA. Combined, for phenotype/genotype correlation 258 cases were available with complete genotypes and complete hand and foot phenotypes.

Table 2 depicts the model fit indices for all models that have been fitted to our data. Table 3 depicts the distribution of phenotypes and genotypes over the two assigned latent classes. Hand and foot phenotypes were used as input for the LCA, thus are all complete cases. Malformation of the cranium and genotypes do have missing cases. Note that for the LCA, full case description was required, resulting in 8 cases due to incomplete phenotypes. Out of these 8, one also had a genotype that thus needed to be excluded. Missing of genotypic data was higher in LC2, mostly due to CNV's (Table 1).

Table 2. Model fit indices for the one-class through six-class model evaluated in our LCA

Number of classes	Log Likelihood	Residual degrees of freedom	BIC	aBIC	cAIC	likelihood ratio	Entropy
1	-1072,0687	57	2178,316	2159,109	2184,316	299,59038	-
2	-966,4844	50	2006,632	1965,407	2019,632	88,42178	0,765
3	-949,9799	43	2013,288	1949,865	2033,288	55,41278	0,740
4	-942,9999	36	2038,993	1953,372	2065,993	41,45279	0,952
5	-937,2077	29	2067,074	1959,255	2101,074	29,86850	0,569
6	-933,5159	22	2099,355	1969,338	2140,355	22,48488	0,716

BIC: Bayesuian information criterion; AIC: Akaike information criterion; LCA: latent class analysis

Table 3. Distribution of phenotypes and genotypes in the two latent classes (LC).

		Latent Class 1 / postaxial phenotype	Latent Class 2 / preaxial phenotype
Number of cases in LC		88	201
Mean Probability of class membership		0.91 (0.88-0.94)	0.96 (0.95-0.97)
Phenotypes		**% of cases in class**	
Hand	Preaxial polydactyly	15,91%	52,74%*
	Postaxial polydactyly	96,59%	40,80%*
	Syndactyly	12,50%	53,73%*
Foot	Preaxial polydactyly	45,45%	95,52%*
	Postaxial polydactyly	69,32%	1,49%*
	Syndactyly	23,86%	83,08%*
Cranium	Macrocephaly	48,33%	33,33%
	Hypertelorism	41,07%	38,42%
	Corpus callosum	18,18%	8,16%
Genotypes		**N cases**	
	Total	85/88	173/201
Included mutations	Frameshift	52	54
	Nonsense	26	42
	Missense	6	54*
	Splice	1	23*

** = $p<0.01$*

In 54/60 cases, a missense variant produced a postaxial phenotype. Likewise, splice site variants show the same phenotype in 23/24 cases (Table 3). For both frameshift and nonsense variants, this relation is not significant (52 preaxial vs. 54 postaxial and 26 preaxial vs. 42 postaxial, respectively). Therefore, only for nonsense and frameshift variants the location of the variant was plotted against the probability for LC2 membership in Figure 1. A full scatterplot of all variants is available in supplementary figure 1.

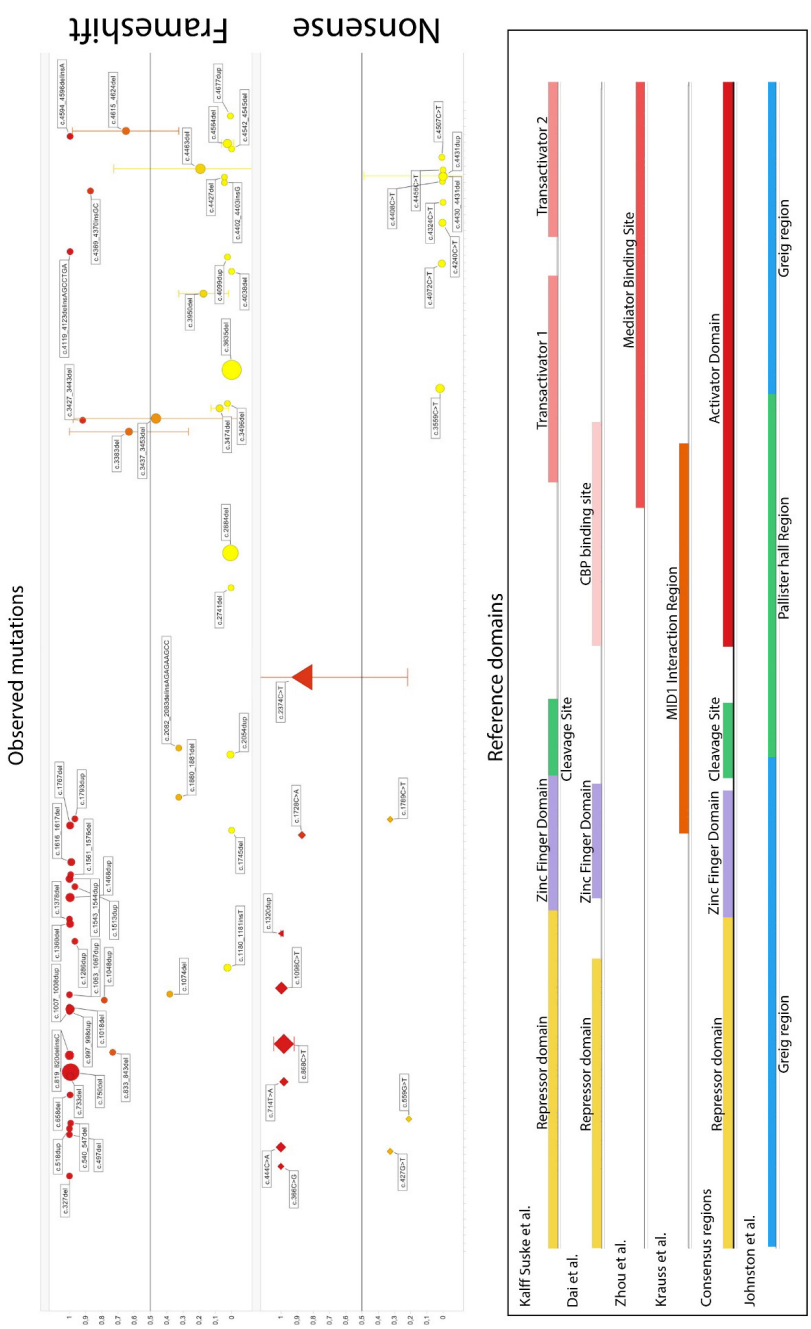

Figure 1.
In this figure the postaxial probability of an anterior phenotype is plotted against the location of the variant, stratified for the type of mutation that was observed. For better overview, only variants with a location effect were displayed. The full figure, including all variant types, can be found in the supplementary figure 1. Each mutation is depicted as a dot, the size of the dot represents the number of observations for that variant. If multiple observations were made, the mean postaxial odds and interquartile range are plotted. For the nonsense variants, variants that were predicted to produce nonsense mediated decay, are depicted using a triangle. Again, the size indicates the number of observations.

Figure 1 reveals a pattern for these nonsense and frameshift variants that reveals that variant at the C-terminal of the gene predict preaxial phenotypes. When relating the domains of the GLI3 protein to the observed phenotype, we observe that the majority of patients with a nonsense or frameshift variant in the repressor domain, the zinc finger domain or the cleavage site had a high probability of a LC2/ preaxial phenotype. This group contains all variants that are either experimentally determined to be subject to NMD (triangle marker in Figure 1) or predicted to be subject to NMD (diamond marker in Figure 1). Frameshift and nonsense variants in the activator domain result in high probability for a LC1/postaxial phenotype. These variants will be further referred to as truncating variants in the activator domain.

The univariate relation of the individual phenotypes to these two groups of variants are estimated and presented in Table 4. In our multivariate analysis, postaxial polydactyly of the foot and hand are the strongest predictors (Beta: 2,548; p<0,001 and Beta: 1,47, p=0,013, respectively) for patients to have a truncating variant in the activator domain. Moreover, the effect sizes of preaxial polydactyly of the hand and feet (Beta: -0,797; p=0,123 and Beta: -1,772, p=0.001) reveals that especially postaxial polydactyly of the foot is the dominant predictor for the genetic substrate of the observed anomalies.

Table 4 shows exploration of the individual phenotypes on the genotype, both univariate and multivariate. The multivariate analysis corrects for the presence for multiple phenotypes in the underlying population.

Table 4. Univariate and multivariate analysis of the phenotype/genotype correlation

			Univariate analysis	**Multivariate analysis**	
			OR frameshift/nonsense mutation '5 side of the zinc finger domain	**Beta**	**p-value**
Phenotype	**Hand**	Preaxial polydactyly	0,27 (CI:0,14-0,54)	-0,797	0,123
		Postaxial polydactyly	12,7 (CI:5,2-31,0)	1,469	0,013
		Syndactyly	0,3 (CI:0,16-0,57)	0,505	0,338
	Foot	Preaxial polydactyly	0,1 (CI:0,032-0,14)	-1,772	0,001
		Postaxial polydactyly	33,9 (CI:15,1-76,0)	2,548	<0.001
		Syndactyly	0,1 (CI:0,054-0,19)	-1,773	<0.001
Regression constant				-0,564	0,729

Although the craniofacial anomalies could not be included in the LCA, the relation between the observed anomalies and the identified genetic substrates can be studied. The prevalence of hypertelorism was equally distributed over the two groups of variants (47/135 vs. 21/47 respectively, $p<0.229$). However for corpus callosum agenesis and macrocephaly, there was a higher prevalence in patients with a truncating variant in the activator domain (3/75 vs 11/41, $p<0.001$; OR 8,8, $p<0.001$). and 42/123 vs 24/48, $p<0,05$). Noteworthy is the fact that 11/14 cases with corpus callosum agenesis in the dataset had a truncating variant in the activator domain

DISCUSSION

In this report, we present new insights in the correlation between the phenotype and the genotype in patients with GLI3-mediated polydactyly syndromes. We illustrate that there are two latent classes of patients, best predicted by postaxial polydactyly of the hand and foot for LC1, and the preaxial polydactyly of the hand and foot and syndactyly of the foot for LC2. Patients with postaxial phenotypes have a higher risk to have a truncating variant in the activator domain of the *GLI3* gene which is also related to a higher risk of corpus callosum agenesis. These results suggest a functional difference between truncating variants on the N-terminal and the C-terminal side of the GLI3 cleavage site.

Previous attempts of phenotype to genotype correlation have not yet provided the clinical confirmation of these assumed mechanisms in the pathophysiology of GLI3-mediated polydactyly syndromes. Johnston et al. have successfully determined the Pallister-Hall region in which truncating variants produce a Pallister-Hall phenotype rather than Greig syndrome.[279] However, in their latest population study, subtypes of both syndromes were included to explain the full spectrum of observed malformations. In 2015, Demurger et al. reported the higher incidence of corpus callosum agenesis in the GCPS population with truncating mutations in the activator domain.[288] Al-Qattan in his review summarizes the concept of a spectrum of anomalies dependent on haplo-insufficiency (through different mechanisms) and repressor overexpression.[289] However, bases this theory mainly on reviewed experimental data. Our report is the first to provide an extensive clinical review of cases that substantiates the phenotypic difference between the two groups that could fit the suggested mechanisms. We agree with Al-Qattan et al. that a variation of anomalies can be observed given any pathogenic variant in the GLI3 gene, but overall 2 dominant phenotypes are present: a population with predominantly preaxial anomalies and one with postaxial anomalies. The presence of pre- or postaxial polydactyly and syndactyly is not mutually exclusive for one of these 2 subclasses; meaning that preaxial polydactyly can co-occur with postaxial polydactyly. However, truncating mutations in the activator domain produce a postaxial phenotype as can be derived the risk in Table 4. The higher risk of corpus callosum agenesis in this population makes that differentiating between a preaxial phenotype and a postaxial phenotype, instead of between the different GLI3-mediated polydactyly syndromes, might be more relevant regarding diagnostics for corpus callosum agenesis.

We chose to use LCA as an exploratory tool only in our population for 2 reasons. First of all, LCA can be useful to identify subgroups, but there is no “true” model or number of subgroups you can detect. The best fitting model can only be estimated based on the available measures and approximates the true subgroups that might

be present. Second, LC-membership assignment is a statistical procedure based on the posterior probability, with concordant errors of the estimation, rather than a clinical value that can be measured or evaluated. Therefore, we decided to use our LCA only in an exploratory tool, and perform our statistics using the actual phenotypes that predict latent class membership and the associated genotypes. Overall, this method worked well to differentiate the two subgroups present in our dataset. However, outliers were observed. A qualitative analysis of these outliers is available in the supplementary data.

The genetic substrate for the two phenotypic clusters can be discussed based on multiple experiments. Overall, we hypothesize two genetic clusters: one that is due to haploinsufficiency and one that is due to abnormal truncation of the activator. The hypothesized cluster of variants that produce haploinsufficiency is mainly based on the experimental data that confirms NMD in two variants and the NMD prediction of other nonsense variants in Alamut. For the frameshift variants, it is also likely that the cleavage of the zinc finger domain results in functional haploinsufficiency either because of a lack of signaling domains or similarly due to NMD. Missense variants could cause haploinsufficiency through the suggested mechanism by Krauss et al. who have illustrated that missense variants in the MID1 domain hamper the functional interaction with the MID1-α4-PP2A complex, leading to a subcellular location of GLI3.[299] The observed missense variants in our study exceed the region to which Krauss et al. have limited the MID-1 interaction domain. An alternative theory is suggested by Zhou et al. who have shown that missense variants in the mediator binding domain can cause deficiency in the signaling of GLI3A, functionally implicating a relative overexpression of GLI3R.[298] However, GLI3R overexpression would likely produce a postaxial phenotype, as determined by Hill et al. in their fixed homo and hemizygous GLI3R models.[291] Therefore, our hypothesis is that all included missense variants have a similar pathogenesis which is more likely in concordance with the mechanism introduced by Krauss et al. To our knowledge, no splice site variants have been functionally described in literature. However, it is noted that the 15th and last exon encompasses the entire activator domain, thus any splice site mutation is by definition located on the 5' side of the activator. Based on the phenotype, we would suggest that these variants fail to produce a functional protein. We hypothesize that the truncating variants of the activator domain lead to overexpression of GLI3R in SHH rich areas. In normal development, the presence of SHH prevents the processing of full length GLI3[283] into GLI3R, thus producing the full length activator. In patients with a truncating variant of the activator domain of GLI3, thus these variants likely have the largest effect in SHH rich areas, such as the ZPA located at the posterior side of the hand/footplate. Moreover, the lack of postaxial anomalies in the GLI3 $^{\Delta 699\,/\,-}$ mouse model (hemizygous fixed repressor model) compared to the GLI3 $^{\Delta 699\,/\,\Delta 699}$ mouse model (homozygous fixed repressor model), suggesting a dosage effect of GLI3R to be responsible for postaxial hand

anomalies.[291] These findings are supported by Lewandowski et al., who show that the majority of the target genes in GLI signaling are regulated by GLI3R rather than GLI3A.[318] Together, these findings suggest a role for the location and type of variant in GLI3-mediated syndromes.

Interestingly, the difference between Pallister-Hall syndrome and GLI3-mediated polydactyly syndromes has also been attributed to the GLI3R overexpression. However, the difference in phenotype observed in the cases with a truncating variant in the activator domain and Pallister-Hall syndrome suggest different functional consequences. When studying Figure 1, it is noted that the included truncating variants on the 3' side of the cleavage site seldomly affect the CBP binding region, which could provide an explanation for the observed differences. This binding region is included in the Pallister-Hall region as defined by Johnston et al. and is necessary for the downstream signaling with GLI1.[44,46,279,319] Interestingly, recent reports show that pathogenic variants in GLI1 can produce phenotypes concordant with Ellis von Krefeld syndrome, which includes overlapping features with Pallister-Hall syndrome.[320] The four truncating variants observed in this study that do affect the CBP but did not results in a Pallister-Hall phenotype are conflicting with this theory. Kraus et al. postulate an alternative hypothesis, they state that the MID1-α4-PP2A complex, which is essential for GLI3A signaling, could also be the reason for overlapping features of Opitz syndrome, caused by variants in MID1, and Pallister-Hall syndrome. Further analysis is required to fully appreciate the functional differences between truncating mutations that cause Pallister-Hall syndrome and those that result in GLI3-mediated polydactyly syndromes.

For the clinical evaluation of patients with GLI3-mediated polydactyly syndromes, intracranial anomalies are likely the most important to predict based on the variant. Unfortunately, the presence of corpus callosum agenesis was not routinely investigated or reported thus this feature could not be used as an indicator phenotype for latent class membership. Interestingly when using only hand and foot phenotypes, we did notice a higher prevalence of corpus callosum agenesis in patients with postaxial phenotypes. The suggested relation between truncating mutations in the activator domain causing these postaxial phenotypes and corpus callosum agenesis was statistically confirmed (OR 8,8, $p<0.001$). Functionally this relation could be caused by the GLI3-MED12 interaction at the MDB: pathogenic DNA variants in MED12 can cause Opitz-Kaveggia syndrome, a syndrome which presentation includes corpus callosum agenesis, broad halluces and thumbs.[321]

In conclusion, there are two distinct phenotypes within the GLI3-mediated polydactyly population: patients with more postaxial and more preaxial oriented hand anomalies. Furthermore, this difference is related to the observed variant in GLI3. We hypothesize that variants that cause haploinsufficiency produce preaxial

anomalies of the hand, whereas variants with abnormal truncation of the activator domain have more postaxial anomalies. Furthermore, patients that have a variant that produces abnormal truncation of the activator domain, have a greater risk for corpus callosum agenesis. Thus, we advocate to differentiate preaxial or postaxial oriented *GLI3* phenotypes to explain the pathophysiology as well as to get a risk assessment for corpus callosum agenesis.

APPENDIX 1.

Structural domains GLI3 gene

Author	Domain	Amino acids
Kalf-Susske et al.[284]	Repressor	1-462
	Zinc Finger Domain	462-645
	Proteolytic Cleavage site	645-748
	TA1	1376-1580
	TA2	1044-1322
Dai et al.[44]	Repressor	1-396
	Zinc Finger domain	480-636
	CBP binding site	826-1132
Johnston et al[46,279,319]	Pallister Hall region	667-1160
Kraus et al. [299]	Repressor	1-397
	Zinc Finger domain	480-632
	Cleavage site	650-750
	Activator domain	827-1132
	MID1-interaction region	568-1100
Zhou et al.[298]	Mediator binding domain	1006-1596

APPENDIX 2.

Included variants in the analysis

Variant	Protein	Type	Observations	Median probability LC2
c.327del	p.Phe109Leufs*50	frameshift	1	0,999
c.497del	p.Pro166Leufs*50	frameshift	1	0,999
c.518dup	p.Ile174Hisfs*2	frameshift	1	0,999
c.540_547del	p.Asn181Cysfs*15	frameshift	1	0,994
c.658del	p.Arg220Valfs*3	frameshift	1	0,996
c.733del	p.Thr245Leufs*65	frameshift	2	0,997
c.750del	p.Tyr251Metfs*59	frameshift	11	0,967
c.819_820delinsC	p.Met274Trpfs*36	frameshift	3	0,999
c.833_843del	p.Arg278Thrfs*22	frameshift	1	0,733
c.997_998dup	p.Tyr334Profs*14	frameshift	1	1,000
c.1007_1008dup	p.Leu337Thrfs*11	frameshift	3	0,996
c.1018del	p.Ser340Valfs*7	frameshift	1	0,999
c.1048dup	p.Tyr350Leufs*62	frameshift	1	0,786
c.1063_1067dup	p.Leu357Serfs*10	frameshift	1	1,000
c.1074del	p.His358Glnfs*7	frameshift	1	0,383
c.1180_1181insT	p.Pro394Leufs*18	frameshift	2	0,025
c.1286dup	p.Met430Aspfs*12	frameshift	1	0,967
c.1360del	p.Gln454Serfs*48	frameshift	2	0,997
c.1378del	p.Val461Serfs*41	frameshift	1	1,000
c.1468dup	p.Glu490Glyfs*14	frameshift	3	0,996
c.1513dup	p.His505Profs*47	frameshift	1	0,967
c.1543_1544dup	p.Arg516Alafs*20	frameshift	2	0,999
c.1561_1576del	p.Ser521Profs*9	frameshift	1	0,994
c.1616_1617del	p.Arg539Thrfs*12	frameshift	1	0,996
c.1617_1633del	p.Arg539Serfs*7	frameshift	1	0,981
c.1745del	p.Gly582Valfs*47	frameshift	1	0,000
c.1767del	p.Asn589Lysfs*40	frameshift	2	0,997
c.1793dup	p.Asn598Lysfs*7	frameshift	1	0,967
c.1880_1881del	p.His627Argfs*48	frameshift	1	0,326
c.2054dup	p.Arg686Alafs*52	frameshift	2	0,006
c.2082_2083delins AGAGAAGCC	p.Val695Glufs*45	frameshift	1	0,326

Variant	Protein	Type	Observations	Median probability LC2
c.2741del	p.Gly914Alafs*38	frameshift	1	0,003
c.2884del	p.Asp962Metfs*41	frameshift	9	0,019
c.3383del	p.Asp1128Alafs*78	frameshift	2	0,632
c.3427_3443del	p.Phe1143Alafs*98	frameshift	1	0,919
c.3437_3453del	p.Leu1146Argfs*95	frameshift	4	0,466
c.3474del	p.Ile1160Phefs*46	frameshift	2	0,073
c.3496del	p.Ser1166Alafs*40	frameshift	1	0,024
c.3635del	p.Gly1212Alafs*18	frameshift	14*	0,005
c.3950del	p.Pro1317Glnfs*102	frameshift	2	0,173
c.4038del	p.Gln1347Argfs*72	frameshift	1	0,001
c.4099dup	p.Ala1367Glyfs*45	frameshift	1	0,024
c.4119_4123delins AGCCTGA	p.Pro1374Alafs*2	frameshift	1	0,996
c.4369_4370insGC	p.Ala1457Glyfs*32	frameshift	1	0,870
c.4402_4403insG	p.Leu1468Argfs*11	frameshift	1	0,043
c.4427del	p.Asn1476Thrfs*12	frameshift	1	0,043
c.4463del	p.Thr1488Lysfs*23	frameshift	4	0,355
c.4542_4545del	p.His1515Profs*3	frameshift	1	0,001
c.4564del	p.Ala1522Profs*2	frameshift	3	0,025
c.4594_4596delinsA	p.Ser1532Thrfs*2	frameshift	1	0,996
c.4615_4624del	p.Thr1539Glyfs*11	frameshift	2	0,654
c.4677dup	p.Gly1560Argfs*38	frameshift	1	0,006
c.1446C>G	p.Cys482Trp	missense	2	0,800
c.1498C>T	p.His500Tyr	missense	3	0,999
c.1559G>A	p.Cys520Tyr	missense	1	0,979
c.1627G>A	p.Glu543Lys	missense	3	0,211
c.1633C>A	p.Pro545Thr	missense	3	0,999
c.1658G>A	p.Cys553Tyr	missense	1	0,870
c.1733G>C	p.Cys578Ser	missense	1	0,996
c.1748G>T	p.Cys583Phe	missense	1	0,870
c.1786C>T	p.His596Tyr	missense	3	0,919
c.1787A>C	p.His596Pro	missense	2	0,999
c.1826G>A	p.Cys609Tyr	missense	11	0,019
c.1873C>T	p.Arg625Trp	missense	7	0,967
c.1874G>A	p.Arg625Gln	missense	4	0,994

Variant	Protein	Type	Observations	Median probability LC2
c.2686G>A	p.Asp896Asn	missense	1	0,999
c.2690C>G	p.Pro897Arg	missense	6	0,996
c.2708C>T	p.Ser903Leu	missense	4	0,994
c.2721C>G	p.Ser907Arg	missense	2	0,997
c.3018C>A	p.Ser1006Arg	missense	4	0,984
c.3534G>C	p.Lys1178Asn	missense	1	0,980
c.366C>G	p.Tyr122*	nonsense	1	0,999
c.427G>T	p.Glu143*	nonsense	1	0,326
c.444C>A	p.Tyr148*	nonsense	4	0,990
c.559G>T	p.Glu187*	nonsense	1	0,211
c.714T>A	p.Tyr238*	nonsense	3	0,980
c.868C>T	p.Arg290*	nonsense	13	0,981
c.1096C>T	p.Arg366*	nonsense	6	0,997
c.1320dup	p.Glu441*	nonsense	1	0,999
c.1728C>A	p.Tyr576*	nonsense	2	0,870
c.1789C>T	p.Gln597*	nonsense	1	0,326
c.2374C>T	p.Arg792*	nonsense	18	0,895
c.3559C>T	p.Gln1187*	nonsense	3	0,019
c.4072C>T	p.Gln1358*	nonsense	2	0,005
c.4240C>T	p.Gln1414*	nonsense	2	0,003
c.4324C>T	p.Gln1442*	nonsense	1	0,001
c.4408C>T	p.Gln1470*	nonsense	1	0,003
c.4430_4431del	p.Ser1477*	nonsense	3	0,000
c.4431dup	p.Glu1478*	nonsense	2	0,000
c.4432G>T	p.Glu1478*	nonsense	1	0,981
c.4456C>T	p.Gln1486*	nonsense	1	0,000
c.4507C>T	p.Gln1503*	nonsense	1	0,006
c.474-2A>G	p.?	splice	5	1,000
c.679+1G>T	p.?	splice	1	0,682
c.679+2_679+15del	p.?	splice	3	0,999
c.827-3C>G	p.?	splice	2	0,434
c.1497+1G>C	p.?	splice	1	0,870
c.1497+1G>A	p.?	splice	2	0,800

Variant	Protein	Type	Observations	Median probability LC2
c.1497+1G>T	p.?	splice	2	0,987
c.1497+2T>G	p.?	splice	3	0,967
c.1498-1G>C	p.?	splice	3	0,996
c.1647+2_1647+3del	p.?	splice	2	1,000

*One case misses complete phenotypic description

APPENDIX 3.

Qualitative analysis of outliers in the phenotype/genotype correlation

Overall the distinction of genotypes based on phenotypes is well defined, however outliers in our analysis were present. Four outliers were identified in the group of truncating variants in the N-terminal side of the gene: c.427 G>T(p.Glu143*), c.559 G>T(p.Glu187*), c.1789C>T(p.Gln597*) and c.2374C>T(p.Arg792*) (Figure 1). Strikingly, the c.2374C>T(p.Arg792*) variant has been experimentally confirmed to produce NMD but produced a variable phenotype. In the case review of patients with this variant, the consensus phenotype of this variant is postaxial polydactyly of the hand, preaxial polydactyly of the foot and syndactyly, thus concordant with the rest of the haploinsufficiency variants. Looking at the effect measures in our regression analysis (Beta's +1,47, -1,77 and -1,77 respectively), this is rightfully classified a preaxial phenotype. The majority of frameshift variants on the 5' side of the cleavage site produced a preaxial phenotype, 3 outliers were observed: c.1074del(p.His358Glnfs*7), c.1180_1181insT(p.Pro394Leufs*18), c.1745del(p.Gly582Valfs*47). The c.1074del(p.His358Glnfs*7) variant was included as a single phenotypic description by the original authors although the variant was present in a larger pedigree. Thus the penetrance of e.g. postaxial polydactyly is unknown but could strongly affect the prediction. The c.1745del(p.Gly582Valfs*47) variant produced a true postaxial phenotype, this variant is located in the zinc finger domain. We hypothesize that the unaffected part of this domain could maintain some function in the produced protein. Frameshift variants on the 3' side of the zinc finger domain, more variability on the phenotype was observed: c.3383del(p.Asp1128Alafs*78), c.3427_3443del(p.Phe1143Alafs*98) ,c.3437_3453del(p.Leu1146Argfs*95), c.4119_4123delinsAGCCTGA(p.Pro1374Alafs*2), c.4369_4370insGC(p.Ala1457Glyfs*32), c.4594_4596delinsA(p.Ser1532Thrfs*2) and c.4615_4624del(p.Thr1539Glyfs*11) all showed a variable or preaxial dominant phenotype. The deletion of multiple nucleotides for most of these variants is noted, however no exact mechanism is apparent for the difference in phenotypic presentation. Alternative splicing could explain the preaxial phenotype, however was not predicted in Alamut. There was 1 missense variant with increased prevalence of postaxial polydactyly, on individual review these were the c.1627G>A(p.Glu543Lys) variants observed in our clinic. This local variant was classified as a variant of unknown significance according to the ACMG guidelines and was observed in all 3 tested cases. Two more family members are symptomatic, but were not tested for this variant. We chose to exclude these 2 unconfirmed cases due to the uncertain pathogenicity of the variant. Nevertheless it is noteworthy that the excluded cases had a preaxial phenotype. Moreover, the single case with a full preaxial phenotype did have abducted, but normally sized, halluces. Further confirmation of this variant is required to confirm its pathogenicity and phenotype.

APPENDIX 4.

Excluded variants

There are a number of variants not included in our analysis that have not been discussed in the manuscript, namely the variants that produced isolated hand or feet phenotypes. Supplementary figure 1 reveals that the included missense variants center around the MID1 interaction region. However, when reviewing the HGMD database, more missense variants are present on the N and C terminal side of the gene. These variants cause isolated preaxial polydactyly and postaxial polydactyly[284,288,313,317,322], but also atrial septal defects, urinary tract anomalies, esophageal atresia and medulloblastoma have been described[323-325]. Missense variants in the N-terminal side of the gene likely produce a non-functional repressor with a functional activator. Since GLI3A seems to have no separate role in the etiology of polydactyly (especially on the postaxial side), the hand phenotype is indeed expected to be comparable to haploinsufficiency. On the other hand, C-terminal missense variants likely hamper the downstage signaling of the activator as suggested by Zhou et al., which as discussed in the manuscript leads to relative repressor overexpression.

8

A point mutation in the pre-ZRS disrupts Sonic Hedgehog expression in the limb bud and results in Triphalangeal Thumb-Polysyndactyly Syndrome

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ABSTRACT

PURPOSE: The zone of polarizing activity regulatory sequence (ZRS) is an enhancer that regulates Sonic Hedgehog (SHH) during embryonic limb development. Recently, mutations in a noncoding evolutionary conserved sequence 500bp upstream of the ZRS, termed the preZRS (pZRS), have been associated with polydactyly in dogs and humans. Here, we report the first case of Triphalangeal Thumb-Polysyndactyly Syndrome (TPT-PS) to be associated with mutations in this region and show via mouse enhancer assays how this mutation leads to ectopic expression throughout the developing limb bud.

METHODS: We used linkage analysis, whole exome Sequencing (WES), Sanger sequencing, fluorescence in situ hybridization, multiplex ligation-dependent probe amplification (MLPA), single-nucleotide polymorphism array and a mouse transgenic enhancer assay

RESULTS: Ten members of a TPT-PS family were included in this study. The mutation was linked to chromosome 7q36 (LOD-score 3.0). No aberrations in the ZRS could be identified. A point mutation in the pZRS (chr7:156585476G>C; GRCh37/hg19) was detected in all affected family members. Functional characterization using a mouse transgenic enhancer essay showed extended ectopic expression dispersed throughout the entire limb bud (E11.5).

CONCLUSION: Our work describes the first mutation in the pZRS to be associated with TPT-PS and provides functional evidence that this mutation leads to ectopic expression of this enhancer within the developing limb.

INTRODUCTION

The genomic landscape of chromosome 7q36 comprises a topological associated domain (TAD)[326], ranging from sonic hedgehog (*SHH*) to limb development membrane protein 1 (*LMBR1*), a gene located ~1 mega base (Mb) upstream of *SHH*[327]. Within this TAD, several regulatory enhancer elements have been identified that are involved in *SHH* regulation during embryonic development of the brain, the central nervous system and the limbs.[328,329] In limb development, *SHH* expression is limited to the Zone of Polarizing Activity (ZPA) in the posterior limb bud, creating a SHH-gradient over the anteroposterior axis of the limb.[330] This gradient is crucial for establishing adequate digit patterning.

The regulation of *SHH* in the embryonic limb bud has largely been attributed to a conserved non-coding regulatory element located in intron 5 of *LMBR1*, called the ZPA Regulatory Sequence (ZRS).[255] Various genetic aberrations of the ZRS have been associated with triphalangeal thumb (TPT) and preaxial polydactyly (PPD) in humans, mice, cats and chickens.[331,332] Molecular studies in polydactylous mice revealed that disruption of the ZRS results in ectopic *SHH* expression in the anterior limb bud.[255]

Point mutations and genomic duplications are the most commonly found ZRS aberrations that lead to limb malformations. Point mutations of the ZRS generally cause triphalangeal thumb accompanied with an additional thumb.[333] Genomic duplications encompassing the ZRS are associated with more severe phenotypes, like Triphalangeal Thumb-Polysyndactyly Syndrome (TPT-PS), Haas-type polysyndactyly or Laurin-Sandrow Syndrome.[334] To date, many point mutations of the ZRS and genomic duplications of different sizes have been reported in the literature.[333,334]

Recently, several studies have suggested that genetic alterations in locations other than the ZRS throughout the 1Mb encompassing *SHH-LMBR1* TAD could be associated with TPT-phenotypes. Petit et al. showed that a 2 kilobase (kb) deletion in a gene desert 240 kb upstream of *SHH*, was linked to familial TPT and hypertrichosis.[335] Additionally, variations in the pre-ZRS (pZRS), a non-coding conserved region approximately 700 base pairs (bp) upstream to the ZRS, were reported in sporadic cases of preaxial polydactyly in humans and dogs.[65,336]

In 1988, Nicolai et al. reported a large Dutch TPT-PS family, consisting of 27 patients.[246] The family pedigree suggested an autosomal dominant inheritance. Subsequently, Tsukurov et al. linked the disease locus in this family to chromosome 7q36 (D7S550, LOD-score: 6.85).[337] Here, we further investigated the molecular causes of this severe limb anomaly in this Dutch TPT-PS family. A whole-genome

SNP-array found linkage between the TPT-PS and the 7q36 locus and, combined with multiplex ligation-dependent probe amplification (MLPA), did not identify any disease associated copy number variations. Karyotyping and Fluorescence in situ hybridization (FISH) did not reveal any specific structural rearrangements within this region. ZRS sequencing and Whole Exome Sequencing (WES) revealed no pathogenic variants. We next sequenced a 4500bp region around the ZRS and identified a point mutation in the pZRS that segregated with the phenotype. Computational analyses did not find increased conservation scores for this mutation compared to previously reported mutations that lead to a much less severe phenotype. A mouse transgenic enhancer essay showed ectopic LacZ expression dispersed through the entire limb bud at E11.5, in both fore and hind limbs. Combined, our study underlines that the integrity of the surrounding environment of the ZRS plays a crucial role in the regulation of *SHH* during limb development.

MATERIALS AND METHODS

TPT-PS family

Two TPT-PS families were identified at the outpatient clinic for Congenital Hand and Upper Limb Anomalies at the Sophia Children's Hospital in Rotterdam, The Netherlands. The family members were consulted and clinically examined by a plastic surgeon and a clinical geneticist. A common ancestor for two Dutch TPT-PS families could be identified in the established pedigree, confirming relatedness of both families (Figure 1). Peripheral blood samples were collected from the affected father, unaffected mother and both affected children in Dutch Family I and the affected mother and child in Dutch Family II. Additionally, the Dutch family reported the presence of Australian relatives with similar congenital hand anomalies. The Australian Family was subsequently consulted and clinically examined and peripheral blood samples were collected from four affected family members in the Australian family. In total, 9 patients with TPT-PS and one unaffected family member were included in this study. Written informed consent was obtained from all family members. This study has been approved by the Medical Ethics Committee of the Erasmus University Medical Centre in Rotterdam the Netherlands (MEC-2015-12).

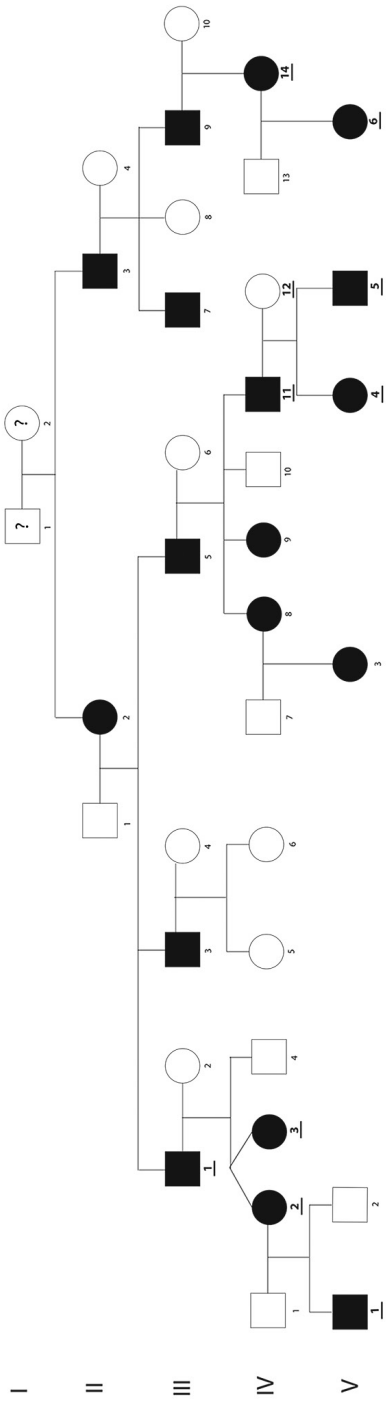

Figure 1. Pedigree of Triphalangeal Thumb-Polysyndactyly Syndrome (TPT-PS) family. Affected family members are identified by filled black squares or circles. The numbers of the 10 family members that were included in this study are underlined and emboldened.

SNP-array and Linkage

Genome-wide genotyping was conducted using 200ng DNA from all included family members with an Illumina Infinium GSAMD-24v1 array (Illumina, San Diego, California; 730,525 SNPs at a median distance of 2.1 kb). The statistical package, easyLINKAGE Plus v5.08 (20), Merlin v1.0.1 software (Abecasis Lab, University of Michigan), was used to perform single-point and multipoint parametric linkage analysis as previously described.[338] Logarithm of odds scores were obtained using a dominant model of inheritance, with 99% penetrance and disease allele frequency of 1:1.000. Furthermore, SNP-array data was additionally used to evaluate the presence of genomic duplications or deletions. Data was analyzed using the Nexus Copy Number, Discovery Edition, Version 7 (BioDiscovery, El Segundo, California).

ZRS Sequencing and Multiplex Ligation-dependent Probe Amplification

Targeted sequencing of the ZRS combined with Multiplex ligation-dependent probe amplification (MLPA) is currently used as standard genetic diagnostic work-up in TPT-families. DNA was isolated from peripheral blood. Fragments were amplified using standard Polymerase Chain Reaction (PCR). An 834bp fragment covering the ZRS was sequenced in all Dutch index patients (chr7:156583766-156584600, GRCh/Hg19).

Additionally, primers were designed to sequence a 4,500bp region surrounding the ZRS (chr7:15681430-156585993, GRCh39, Hg19). A total of 8 primer pairs were required in order to cover this entire sequence (Table S1.).

The PCR products were sequenced using Big Dye Terminator 3.1. The fragments were loaded on an Abi 3130 Sequence analyzer and genetic analysis was performed with SeqScape Software (v3.0).

Multiplex Ligation-dependent Probe Amplification (MLPA) analysis (MRC Holland S134 kit) was used to detect the presence of genomic duplications and deletions between exon 3 and exon 6 of LMBR1, encompassing the ZRS.

Karyotyping and Fluorescent In Situ Hybridization (FISH)

Karyotyping was performed on GTG-banded metaphases obtained from peripheral blood cultures using standard procedures. Karyotypes were obtained from V-6 and a control. Results were described in accordance with the ISCN 2005.[339] FISH

was carried out on the fixed sediments of the karyotyping cultures of subjects V-6 and a control. FISH analysis was performed using five 7q36 located bacterial artificial chromosome (BAC) clones. The BAC clones (RP11-773c4, RP5-982e9, RP11-332e22, RP11-51l24 and RP11-50D7, Table S2) were selected from the University of Santa Cruz (UCSC) genome browser (UC Santa Cruz, Santa Cruz, CA, USA; assembly March 2006) and ordered from BACPAC Resources (Children's Hospital of Oakland Research Institute, Oakland, CA, USA). After isolation of the BAC DNA, the probes were labeled and used for FISH on chromosome preparations from patients and parents, according to standard protocols.[340]

Whole Exome Sequencing

Samples of patient IV-2, V-1, V-4 and V-5 were sequenced using an Illumina Nextseq500 machine. The exome samples were captured using the Truseq Exome Library Prep Kit and the Illumina TruSeq Exome Library Prep Reference Guide v01. Reads were aligned against the Human Reference Genome build 19 (hg19) and genetic variants were called using the Qiagen CLC genomics workbench. The Variant Call Format (.vcf) files were annotated with Annovar.[341] Subsequently, the annotated data was filtered using the previously obtained linkage data, the presence of the variant in all 4 members of the family and an allele frequency of less than 0.001 in publicly available reference datasets, including 1000 genomes[342], ExAC[343] and Qiagen HGMD professional.[344]

Mouse Enhancer Assay

A genomic region encompassing both pZRS and ZRS (chr7:156583545-156585773, GRCh37/hg19, 2229 base pairs) with either the reference sequence or the chr7:156585476G>C change was cloned into an HSP 68-Lac Z vector (Addgene #37843) by carrying out a PCR on genomic DNA from patient V-5. The cloned plasmids were sequenced verified to contain the mutation allele and the wildtype allele and to exclude any other variants. Transgenic mouse E11.5 embryos followed by beta-galactosidase staining were generated by Cyagen Biosciences. Pictures of embryos were taken using a Leica M205FA stereomicroscope and annotated independently by multiple curators based on the observed spatial expression pattern. All mouse work was approved by the UCSF institutional animal care and use committee.

RESULTS

Clinical Report

All affected family members had a phenotype that corresponds with Triphalangeal Thumb-Polysyndactyly Syndrome (TPT-PS, OMIM: 174500). The pedigree demonstrated an autosomal dominant inheritance of the phenotype. All patients were bilaterally affected. They presented with at least one triphalangeal thumb on both hands. The hands typically demonstrated a poly- and syndactylous block of digits on the preaxial and postaxial side, with the second and occasionally the third digit present between both blocks. Additionally, postaxial syndactyly and polydactyly of both feet were observed in patients V-4, V-5 and V-6. The affected family members did not present with other congenital anomalies. (Figure 2, S3 3)

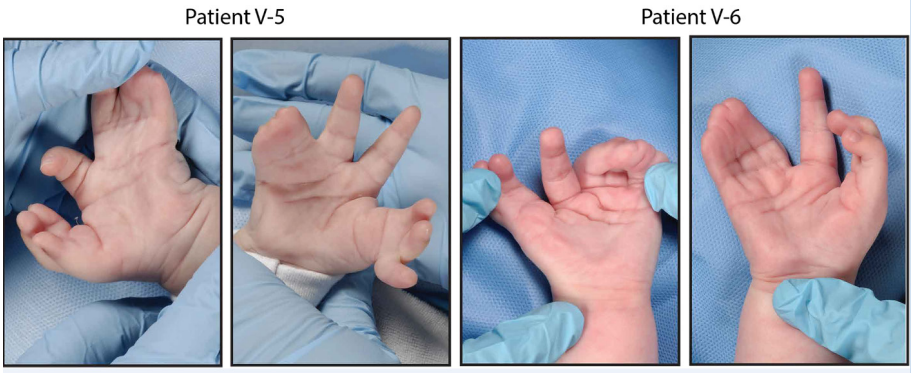

Figure 2. Clinical images of Triphalangeal Thumb-Polysyndactyly Syndrome (TPT-PS) phenotypes of patients V-5 and V-6.

TPT-PS is linked to 7q36

Genome wide linkage analysis was performed to validate the linkage results that were obtained by Tsukurov et al.[337] With SNP-array data of all 10 included family members, LOD-Scores were calculated using easylinkage software. Linkage was confirmed at chromosome 7q36 (LOD-score 3.0, chr7:156033299-158113390, GRCh37/hg19). This region corresponds with the region that was found in the same TPT-PS family by Tsukurov et al. (LOD-score, D7S550).[337] Additionally, a maximum LOD-score of 3.0 was found on chromosome 21q22.11 (chr21:33405700-36673573, GRCh37/hg19).

Absence of ZRS and copy number disease associated variations

As the ZRS has been associated with various limb malformations, we analyzed our family for mutations and copy number variations (CNVs) in this region. The ZRS was sequenced in patients V-4, V-5 and V-6 and did not reveal any disease associated alterations. As TPT-PS is commonly associated with duplications including the ZRS[334], SNP-array data of all TPT-PS patients were reviewed to identify CNVs on chromosome 7q36. No copy number variations larger than 2.1kb were found on chromosome 7q36 (Figure 3.) or chromosome 21q22.11. Finally, Multiplex Ligation-dependent Probe Amplification (MLPA) analysis did not reveal pathogenic deletions or duplications between exon 3 and exon 6 of *LMBR1.*

Karyotyping using GTC banded chromosomes revealed no aberrant chromosomal pattern in the two tested individuals. FISH with dual-labeled BAC probes using different probe combinations (Figure 3, S4) excluded small sub-chromosomal rearrangements in the *SHH-LMBR1* region or the telomeric part of chromosome 7q36. Also, using a BAC probe covering the ZRS region in intron 5 of the *LMBR1* gene (RP11-51I24) we did not detect any aberrant patterns of hybridization in interphase nuclei or banded chromosomes.

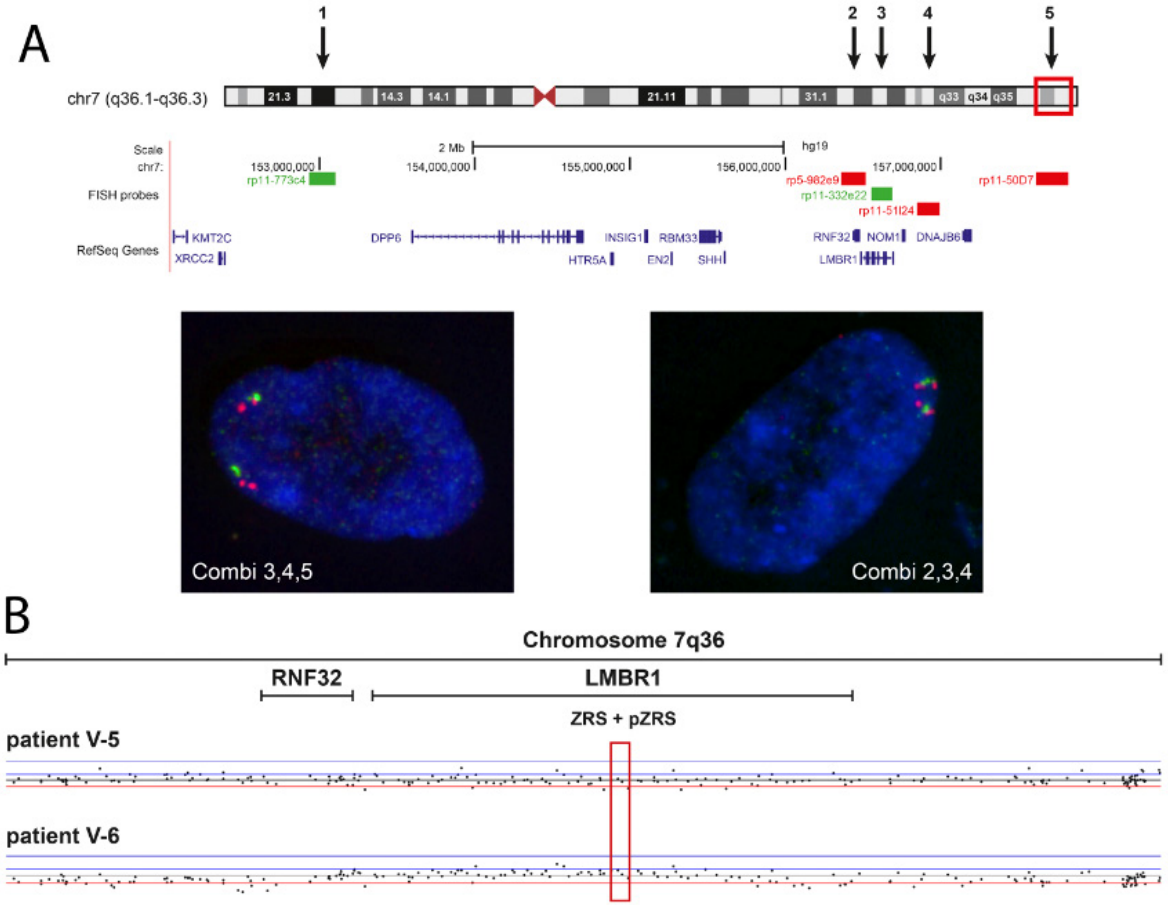

Figure 3. A. *Results of Fluorescence in Situ Hybridization (FISH) analysis of patient V-5. The location of 5 FISH-probes are depicted in the genome track. The labeled colors of each probe correspond with the color displayed on the images.* ***B.*** *Images of Nexus Copy Number analysis (Illumina Infinium GSAMD-24v1 microarray) in patients V-5 and V-6 do not show copy number variations on chromosome 7q36.*

No pathogenic exonic variants linked to limb defects

We next wanted to test whether coding mutations in other genes might be associated with this phenotype. Samples of four affected patients were investigated with Whole Exome Sequencing (WES) to verify if an exonic pathogenic variant was present on chromosome 7q36, 21q22.11 or in other coding regions. The affected family members did not share any predicted pathogenic variants with an allele frequency of less than 0.001 in 1000 genomes[342], ExAC[343] and HGMD[344] datasets.

Identification of a point mutation in the pZRS

After sequencing a 4.5kb region covering the ZRS and pZRS, a heterozygous G>C point mutation in the pZRS (chr7:156585476G>C; hg19) was identified (Figure 4). This point mutation was found in all affected patients and was absent in an unaffected family member. The variant was not present in online databases (dbSNP[345], ClinVar[346], ExAC[343] and HGMD) or in locally available WGS datasets (GoNL[347] and Wellderly[60]).

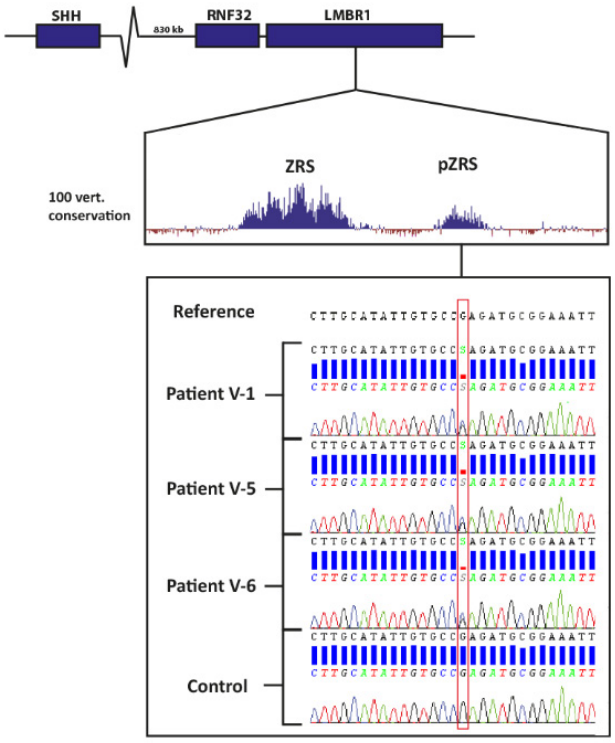

Figure 4. *Mutation location of the identified point mutation in intron 5 of LMBR1. The pZRS is visualized through the conservation track in the UCSC genome browser. The chromatogram of 3 affected family members reveal a G>C point mutation.*

Computation analyses of pZRS variants

Previous reported mutations in the pZRS were found to only lead to preaxial polydactyly in dogs[336] and humans.[65] However, our identified pZRS mutation, chr7:156585476G>C, leads to a much more severe phenotype, TPT-PS, which also includes the lower limbs. To test for potential causes for these phenotypic differences, we carried out various computational analyses on these different mutations. Evolutionary conservation analyses of this variant compared to the previously discovered canine and human variants using both PhyloP and GERP[348] did not find chr7:156585476G to have higher conservation scores than these previously reported variants (Figure S5). Additionally, Combined Annotation Dependent Depletion (CADD) scores[349] for this nucleotide were not higher than these other variants (Figure S5). Combined, these analyses could not explain the cause for the stronger limb phenotype observed in this family.

Furthermore, TPT-PS is widely associated with genomic duplications that cover the ZRS. Duplications that include the ZRS are also able to cause more severe anomalies, like Haas-type Polysyndactyly and Laurin-Sandrow Syndrome.[334] The similarity of phenotypes in pZRS mutations and genomic duplications indicate corresponding levels and patterns of SHH disruption in the limb bud. This similarity also introduces the hypothesis that *SHH* regulation in pZRS mutations and genomic duplications might be affected by the same underlying molecular mechanism.

Although genomic duplications encompassing the ZRS have regularly been reported in the literature, no study has investigated the pathogenic mechanism that ultimately results in these severe limb anomalies. Two hypotheses have been suggested regarding genomic duplications of the ZRS. First, it has been proposed that a genomic duplication could have a dosage effect, affecting the regulatory balance between enhancers and promoters.[350]

Second, genomic duplications are able to alter the chromatin organization of the entire genome.[351] Hi-C sequencing has revealed that the chromatin structure is partitioned into various Topological Associated Domains (TADs). Duplications of the ZRS therefore might disrupt a TAD-boundary in the vicinity of the ZRS and cause limb anomalies through one of these mechanisms. Through the Hi-C 3D-genome browser and ENCODE track for chromatin organization, we mapped the predicted TAD-boundaries and CTCF and RAD21 binding sites in the SHH-ZRS domain.[352,353](Figure S6) The Hi-C genome browser and the presence of increased CTCF and RAD21 binding sites predicted a TAD-boundary in intron 4 of *LMBR1*, 25 kb upstream of the ZRS. Lohan et al. reported 16 different sizes of genomic duplications encompassing the ZRS that caused severe limb anomalies. 15 of these duplications also include the predicted TAD boundary in intron 4 of *LMBR1*. However, the smallest duplication

of 16kb in a Laurin-Sandrow Syndrome family did not surpass this TAD boundary. As the phenotypes of genomic duplications and point mutations in the pZRS have close similarities, we assessed if the pZRS represents an additional TAD-boundary that can be disrupted by point mutations. We did not identify increasing CTCF and RAD21 binding sites in the pZRS region (Figure S6).

Therefore, although the disruption of the TAD-boundary in the vicinity of the ZRS-complex could explain the pathogenic mechanism in TPT-PS families with genomic duplications, it is not able to elucidate the mechanism in the family with a point mutation in the pZRS or the previously reported family with the 16kb duplication.

chr7:156585476G change leads to ectopic ZRS-expression in the developing limb bud

We next carried out mouse transgenic enhancer assays on both the reference and mutant allele to test whether it leads to any altered enhancer activity. Both the pZRS and ZRS region for each allele were cloned into a mouse enhancer assay vector and analyzed for their LacZ expression at E11.5 in transgenic mice. For the reference allele, 8 transgenic assays were made, of which 7 showed expression in the posterior margin of the limb bud (Figure 5,S7). We observed a significant expansion of LacZ expression for the TPT-PS associated allele, extending to the entire mesenchyme all the way into the trunk region in 5 out of 6 mutated transgenic enhancer assays made (Figure 5, S7). In addition, the mutant allele also showed LacZ expression in the mandibular process that was not observed in the reference sequence. It is worth noting that both alleles also showed consistent LacZ staining in the snout, similar to that previously observed for the canine sequence[336] and that the reference allele showed slight expression in the anterior autopod (Figure 5, S7) different from previous studies that tested ZRS only[354] or the canine ZRS+pZRS in mice.[336] In sum, our results show that transgenic mice carrying the mutated pZRS allele have increased enhancer activity dispersed throughout the entire limb bud including the trunk region at the base of the limb compared to the reference allele.

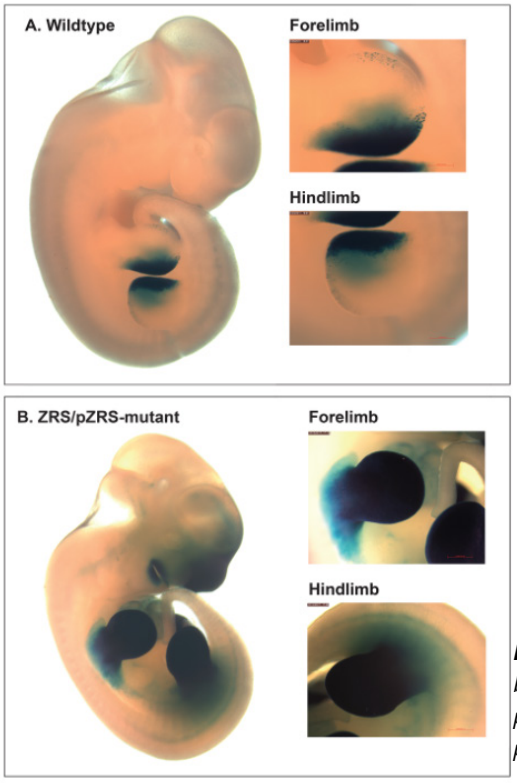

Figure 5. *LacZ expression in mice embryo's (E11.5) carrying the wildtype ZRS/pZRS construct (6A) or the mutated ZRS/pZRS construct (6B).*

DISCUSSION

In this study, we report a Dutch family with Triphalangeal Thumb-Polysyndactyly Syndrome with a point mutation in a conserved non-coding regulatory region in the genome; the pZRS. This point mutation was identified after several abnormalities known to cause a TPT-PS phenotype were ruled out, including point mutations, copy number variations and chromosomal rearrangements of the ZRS as well as pathogenic variants in other genes. We discovered a novel mutation in the pZRS that segregated with the disease and showed differential enhancer activity in transgenic mice.

Previously reported pZRS mutations

Two studies have previously associated point mutations in the pZRS with congenital limb anomalies. Park et al. identified two point mutations in the pZRS in two different breeds of dogs[336], but did not find altered expression in an enhancer assay. However, it is important to also note that this mutation is located near the telomeric end of the pZRS (Figure S5). In another study, Xiang et al. screened a large population of children with non-syndromic preaxial polydactyly for mutations in *SHH*, *GLI3*, ZRS and pZRS.[65] Patients with isolated preaxial polydactyly of the thumb are often unilaterally affected and do not have a positive family history. Therefore, it is questionable whether this phenotype is due to underlying genetic aberrations. In addition, these mutations were not followed up via an enhancer assay for their functional consequences. Computational analyses of our identified mutation did not show increased conservation scores, which could have potentially explained the more severe phenotype.

The role of the pZRS in this SHH-ZRS complex

The functional role of the pZRS remains largely unknown. The pZRS was delineated from the ZRS by Park et al. and is well conserved among vertebrate species and positioned approximately 750bp 5' upstream of the ZRS. Until the present study, there was no functional or molecular evidence confirming that the pZRS is an independently functioning regulatory element. Considering the close proximity of the pZRS and the ZRS, the pZRS could potentially function as an independent regulatory element of *SHH* but also could function alongside the ZRS.

The central part of the ZRS has previously been shown to have a role in regulating the levels of SHH expression in the limb[355], with the 5' end involved in spatiotemporal functions and the 3' end required for long range activity of the ZRS that ensures SHH signaling.[355,356] Additionally, different ETV sites throughout the

ZRS govern the posterior restriction of SHH to the ZPA.[357] Further molecular and functional studies will be required in order to determine which regulatory role the pZRS has and how it is associated with SHH limb expression.

Genotype-Phenotype Correlation

The observation of severe TPT-phenotypes in combination with a single pZRS point mutation in this family provides insights into the possible underlying pathogenic mechanism of this region. The TPT-PS phenotype is clearly more severe than TPT phenotypes caused by previously described point mutations in the ZRS. Several studies have shown that point mutations in the ZRS affect the activity of the ZRS by inserting or removing transcription factor binding sites (TFBS) of ETS, ETV, HOX, and TFAP2B.[355,357,358] These point mutations generally lead to isolated TPT or TPT with one additional thumb. Other described point mutations in the ZRS, however, do cause more severe anomalies than isolated TPT with polydactyly, such as those involving positions 402 and 404 that are associated with isolated TPT with tibial hypoplasia.[255,259,359] In contrast to our reported family, the severe anomalies in 402 and 404 point mutations are only observed in the lower limb and not in the upper limb.

A recent study also revealed that a point mutation in position 105 causes severe TPT-phenotypes that resemble TPT-PS in several affected family members of a large Dutch TPT population.[70] However, the simultaneous presence of less severe phenotypes such as isolated TPT and TPT with preaxial polydactyly in other family members indicate that additional non-genetic factors or genetic modifiers are required to magnify SHH disruption in the limb bud. Considering the severe phenotype in the present Dutch family, it is unlikely that alterations in TFBS affinity have the ability to induce the level of disruption that is required to cause consistent TPT-PS phenotypes in this family. Furthermore, as TPT-PS is mainly observed in families with genomic duplications that encompass the ZRS, we evaluated whether pZRS mutations can alter the integrity of TAD-boundaries in the *SHH-LMBR1* topological domain. We did not identify increased presence of CTCF or RAD21 binding sites in the pZRS. Additionally, the TAD boundary seems to be located approximately 25kb from the telomeric end of the pZRS. (Figure S6) Therefore, pZRS mutations are not likely to be involved in the disruption of the *SHH-LMBR1 TAD*.

Altered enhancer activity

Our mouse enhancer assay that encompassed both the ZRS and pZRS showed enhancer expression beyond the normal posterior ZPA expression pattern of previous transgenic mice carrying the human ZRS sequence[354] or the dog

ZRS+pZRS sequence.[336] For the dog mutations, similar mouse transgenic enhancer assays did show a slight expansion of enhancer activity in the posterior limbs compared to the reference sequence.[336] In our study, mice carrying the reference allele did show slight anterior expression, but those carrying the mutated allele showed significantly more extended enhancer expression. This extended ectopic expression pattern could explain the severity of the limb phenotype in this family and suggests that the pZRS is involved in SHH expression and limb embryonic development and that mutations in this region can cause a severe Triphalangeal Thumb-Polysyndactyly phenotype.

The regulatory network of *SHH* remains to be completely elucidated. New initiatives combining information from the molecular, genetic and clinical points of view will help to elucidate the complex regulatory network in this locus that may also serve as a model for understanding long range regulatory mechanisms for other loci.

Assay name	Gene*	Primer	FR	Build*	Chr	START Position *	Strand	Oligo Type	Assay type	Initial Dectection	Target	Species	Scale	Mod'5	Mod'3	Purifi-cation	Remarks
ZRS2	LMBR1	TCAAACAATCTTACTGCCTTTGATGTG	F	hg19	7	156582148	plus	Oligo	VAR	SEQ	DNA	Human	25nm	No	No	STD	
ZRS2	LMBR1	GCAAAGTGACATGAAAAATTACATTGG	R	hg19	7	156582886	plus	Oligo	VAR	SEQ	DNA	Human	25nm	No	No	STD	
ZRS3	LMBR1	AAACAGAGGCCTGAACTATCAAACATT	F	hg19	7	156582686	plus	Oligo	VAR	SEQ	DNA	Human	25nm	No	No	STD	
ZRS3	LMBR1	CTAGGAGGGCACTAAATTATGGCTTT	R	hg19	7	156583385	plus	Oligo	VAR	SEQ	DNA	Human	25nm	No	No	STD	
ZRS4	LMBR1	TCAATACTGAGAGGCAAAGATGAACA	F	hg19	7	156583235	plus	Oligo	VAR	SEQ	DNA	Human	25nm	No	No	STD	
ZRS4	LMBR1	ACAAAAGGGAAGCCCCTGCT	R	hg19	7	156583909	plus	Oligo	VAR	SEQ	DNA	Human	25nm	No	No	STD	
ZRS5	LMBR1	TCTTGAAGGTGTTGGGAAAATCAA	F	hg19	7	156583767	plus	Oligo	VAR	SEQ	DNA	Human	25nm	No	No	STD	
ZRS5	LMBR1	GCGTAGCACACGGTCTGTAGGA	R	hg19	7	156584466	plus	Oligo	VAR	SEQ	DNA	Human	25nm	No	No	STD	
ZRS6	LMBR1	CGCTTCCACCTGGTCAGTCC	F	hg19	7	156584333	plus	Oligo	VAR	SEQ	DNA	Human	25nm	No	No	STD	
ZRS6	LMBR1	GGCTCTTAGGGCTCAGGGAAC	R	hg19	7	156584950	plus	Oligo	VAR	SEQ	DNA	Human	25nm	No	No	STD	
ZRS7	LMBR1	TTTCTGGAGTGGAGGAGGGAGA	F	hg19	7	156584850	plus	Oligo	VAR	SEQ	DNA	Human	25nm	No	No	STD	
ZRS7	LMBR1	CCTGGGCAATCGATGTTCAA	R	hg19	7	156585518	plus	Oligo	VAR	SEQ	DNA	Human	25nm	No	No	STD	
ZRS8	LMBR1	GATTTTCAATTCAAGCTCTGCATCC	F	hg19	7	156585421	plus	Oligo	VAR	SEQ	DNA	Human	25nm	No	No	STD	
ZRS8	LMBR1	GCATACATTTGAGCCCTTTCATGTT	R	hg19	7	156585993	plus	Oligo	VAR	SEQ	DNA	Human	25nm	No	No	STD	
ZRS1A	LMBR1	ATTTCTTCTTCCTCTGTGCTACG	F	hg19	7	156581787	plus	Oligo	VAR	SEQ	DNA	Human	25nm	No	No	STD	60 C design
ZRS1A	LMBR1	CGTTTGTCAATATATAACTGAACCTTG	R	hg19	7	156582267	plus	Oligo	VAR	SEQ	DNA	Human	25nm	No	No	STD	60 C design
ZRS1B	LMBR1	AGGAGATCTGAGAATCATTTGG	F	hg19	7	156581430	plus	Oligo	VAR	SEQ	DNA	Human	25nm	No	No	STD	60 C design
ZRS1C	LMBR1	AAATATTTATTGGTTTGCACATTTTAC	R	hg19	7	156582067	plus	Oligo	VAR	SEQ	DNA	Human	25nm	No	No	STD	60 C design

Table S1. Details on primer design for sequencing the 4.5kb region encompassing the ZRS + pZRS

Probe number	Rpname	start HG19	end HG19	distance to next probe
5115	rp11-773c4	152928697	153092756	3258617
6983	rp5-982e9	156351373	156509591	36958
1477	rp11-332e22	156546549	156689378	147298
1591	rp11-51I24	156836676	156985055	620482
3108	rp11-50D7	157605537	157815858	

Table S2. Details on design of the Fluorescence in Situ Hybridization (FISH) probes

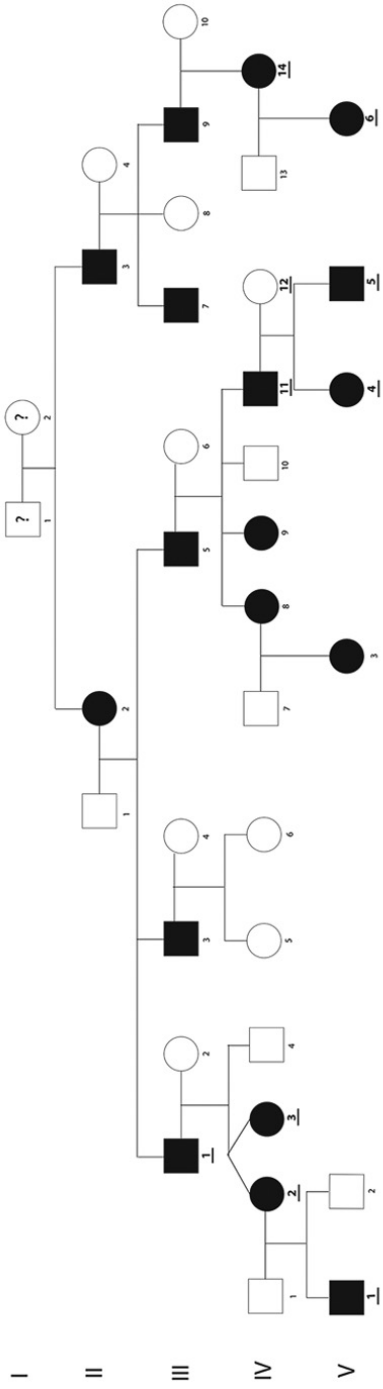

Figure S3. Clinical images of affected family members. Pre-operative images are only available for patients V-4, V-5, V-6. The clinical images of patient III-5 and IV-14 can be found in the publication of Nicolai et al. (Figure 2 and 3).

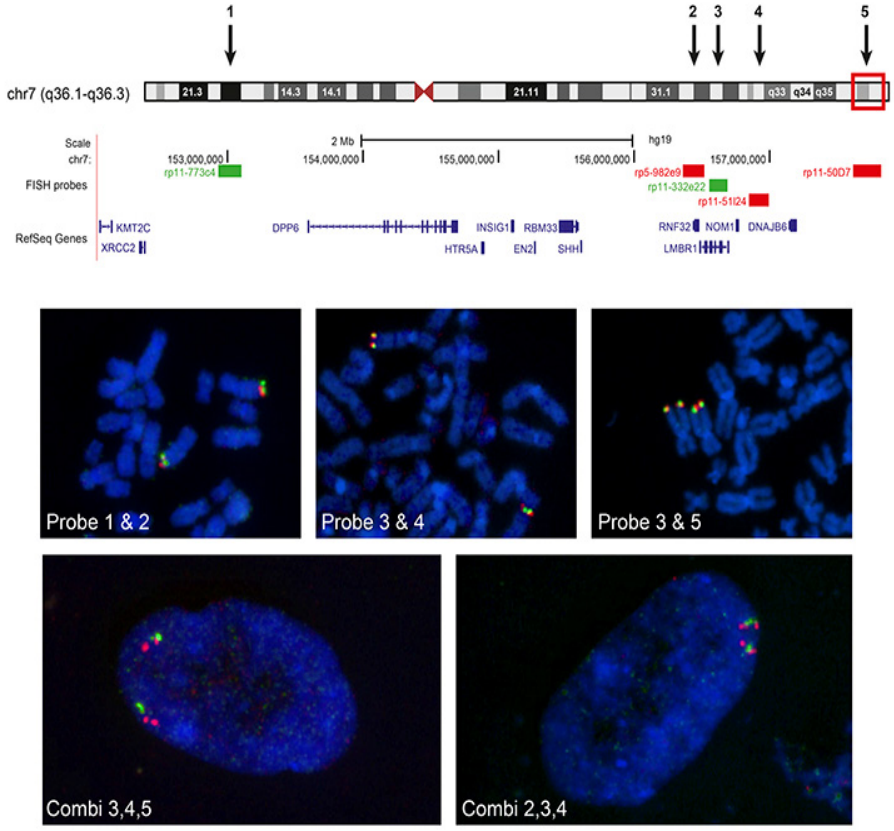

Figure S4. Results of Fluorescence in Situ Hybridization (FISH) analysis in patient V-5. Karyotyping was performed on GTG-banded metaphases and interphases. The location of 5 FISH probes are depicted in the genome track (Table S2). The labeled colors of each probe correspond with the color displayed on the images.

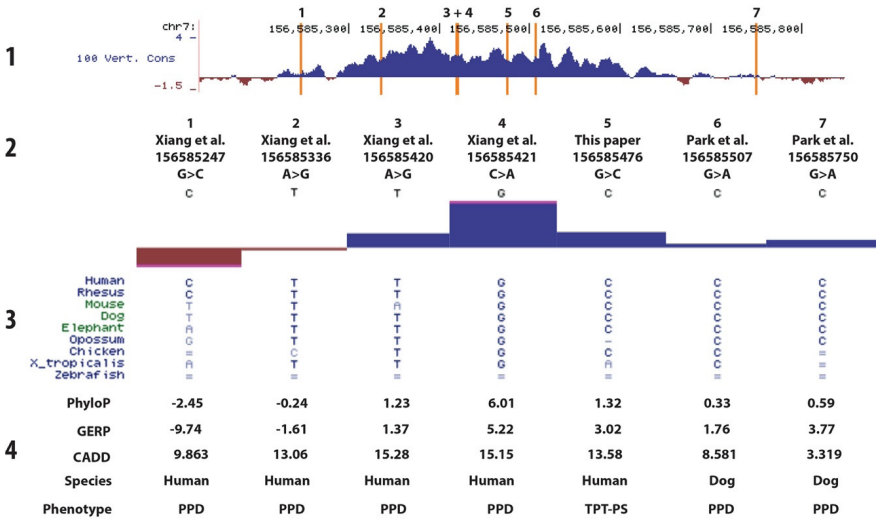

Figure S5. Overview of other reported mutations in the pZRS. From top to bottom: 1) The conservation track among 100 vertebrates as displayed in the UCSC genome browser 2) Author and genomic location of observed point mutations in the pZRS (hg19) 3) nucleotide at specific location in various species. 4) Overview of the corresponding PhyloP, GERP, CADD scores, species and phenotype associated with the specific mutation.

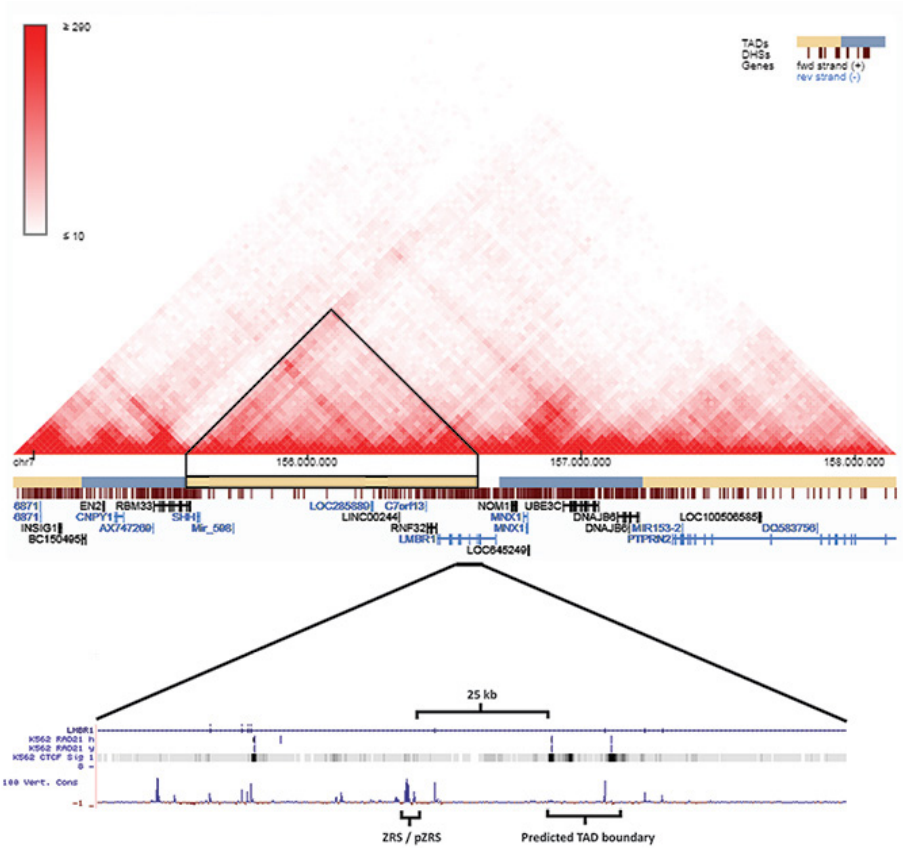

Figure S6. The SHH-LMBR1 topological associated domain (TAD). The TAD boundary in LMBR1 is approximately 25kb away from the ZRS-complex, suggesting no involvement of the point mutation in the pZRS in TAD-boundary disruption.

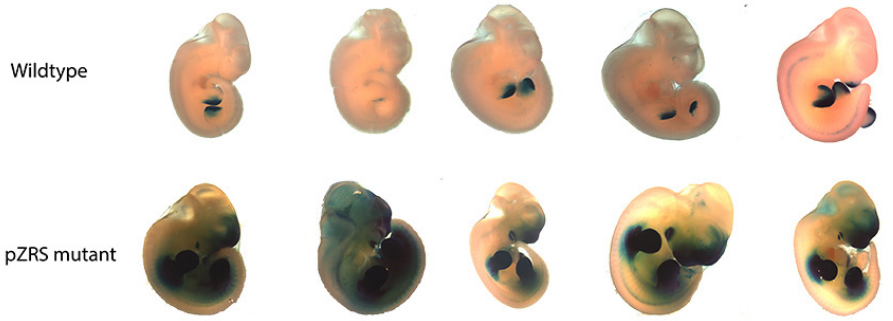

Figure S7. LacZ expression of mice embryo's (E11.5) carrying the wildtype ZRS/pZRS construct and the mutated ZRS/pZRS construct.

9

Clinical and Genetic evaluation of a cohort of patients with Radial Longitudinal Deficiency

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Manuscript in preparation

ABSTRACT

PURPOSE: Although in the early work of Temtamy and McKusick the association between RLD and several syndromes has been discussed elaborately, little data is available on the presentation and genetic evaluation of RLD patients in the clinic. Therefore, this study describes a historical cohort of RLD cases and reviews the clinical presentation, diagnosed syndromes, and molecular diagnostics. Subsequently, different sub-groups of RLD patients were contacted and were offered new molecular evaluation using NGS analyzed using both a gene panel and trio analysis.

METHODS: A historical cohort of patients from 1996 till 2018 was included. Clinical presentation, syndrome diagnoses, and molecular work-up were reviewed. Patients and parents of patients with bilateral occurrence or multiple congenital anomalies without a confirmed molecular diagnosis were offered further analysis using next generation sequencing, using either WES and WGS.

RESULTS: 188 patients with RLD were reviewed, 118 patients with bilateral anomalies, 71 unilateral. In 139 out of 188 patients, at least one other congenital anomaly besides the upper limb anomalies was observed. Compared to a general population of CULA, patients with an RLD have a higher risk of bilateral occurrence, associated anomalies, and an underlying syndrome, including: VACTERL association n=38, Holt-Oram syndrome n=17, TAR syndrome n=7, Goldenhar n=3, Fanconi anemia n=3. Clinical predictors of these syndromes were evaluated. Twenty-six patients were included for further genetic evaluation using NGS, grouped phenotypes included: isolated limb anomalies (n=9), Holt-Oram-like (n=8), VACTERL like (n=7), and two patients with RLD combined with urogenital anomalies. Sequencing revealed multiple pathogenic variants, including variants in genes not priory related to RLD and new candidate genes for RLD related syndromes.

CONCLUSION: This study provides clinical features for referral and guides the evaluation and counseling of these patients and their families. Genome wide sequencing revealed pathogenic variants genes that are not commonly related to RLD; these variants would be missed in targeted diagnostics. Four new candidate genes for RLD were established; *DOCK6*, *GLI2*, *TBX6*, and *NOP2*. We advocate using NGS in RLD patients.

INTRODUCTION

Radial longitudinal deficiency (RLD) represents a spectrum of upper limb anomalies originating from the disruption of the radio-ulnar axis development of the upper extremity. The presentation varies from hypoplasia of the thumb and wrist to "radial club hand" / radial dysplasia with or without abnormal humeral development[360]. Although in the early work of Temtamy and McKusick the association with several syndromes has been discussed elaborately, little data is available on the presentation and genetic evaluation of RLD patients in clinic[13]. The available data suggests RLD has a higher bilateral occurrence and more often presents associated anomalies than other congenital upper limb anomalies[2,361].

Although multiple syndromes are related to RLD, data on the prevalence of the clinically diagnosed syndromes in this population and the subsequent yield of genetic screening is limited[362-364]. Recently, extensive chart reviews of American and Japanese RLD cohorts revealed that up to 68% of cases have associated anomalies, the four most diagnosed syndromes/associations being VACTERL, Holt-Oram, TAR, and Fanconi anemia[365]. Although this data provides reasonable estimates for the clinical evaluation of these patients, it lacks data on the genetic diagnostics applied. Furthermore, the presentation of historical cohort studies of rare conditions implies that some cases might have been clinically diagnosed in an era that did not allow for genetic evaluation to current standards of diagnostics. Therefore, it is hard to extrapolate these numbers to the primary consultation and evaluation of an RLD patient in the clinic.

Alternatively, case series of molecularly confirmed diagnoses are available, such as for Holt-Oram syndrome; Vanlerberghe et al. illustrate that out of the 105 cases with a clinical Holt-Oram diagnosis, 61 could be molecularly confirmed with a pathogenic *TBX5* variant[366]. Moreover, in the total 78 confirmed cases, 17 did not meet the diagnostic criteria for Holt-Oram syndrome. These results illustrate that this yield cannot be easily transferred to a general RLD population and suggests a broader indication for *TBX5* evaluation than the clinical diagnosis Holt-Oram.

Next generation sequencing enables the physician to evaluate a wide variety of genes and thus provides a genetic diagnosis that is unbiased by our prior clinical evaluation. This could benefit RLD patients since over 1000 genes have been related to congenital upper limb anomalies[25]. Especially in patients considered to fit a clinical syndrome diagnosis, e.g., Holt-Oram syndrome, in whom no *TBX5* mutation can be detected. On the other hand, this also implies that there is a risk for incidental findings. Different filters might reduce this risk and conflict with the diagnostic yield: e.g., BRCA2 variants might be incidental, but the gene cannot be disregarded due to its role in Fanconi anemia[367].

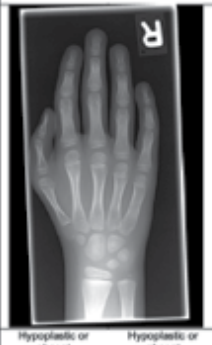
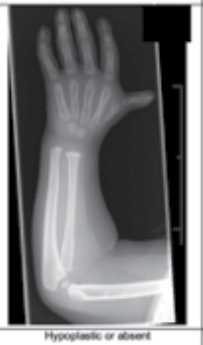
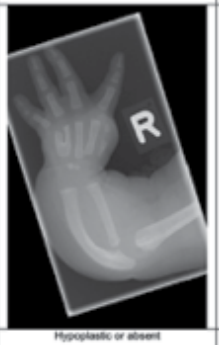
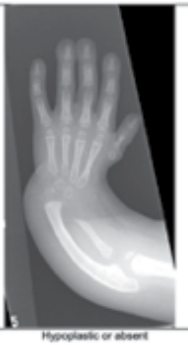
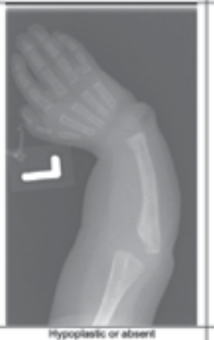
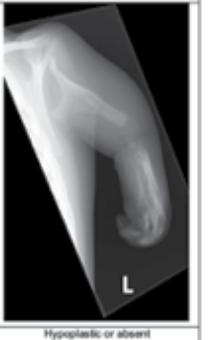
To aid counseling and diagnostic evaluation of RLD patients in clinic, this study describes our historical cohort of RLD cases and reviews the clinical presentation, diagnosed syndromes, and molecular diagnostics. Subsequently, different groups of RLD patients were contacted and were offered new molecular evaluation using NGS analyzed using both a gene panel and trio analysis.

METHODS

Patients selection and data collection

A historical cohort of patients visiting the Sophia Children's Hospital with RLD from 1996 till 2018 was reviewed. A complete chart review of both clinical and genetic records was performed for each case. All radiographs were re-evaluated, and the severity of the deformity was classified according to the modified Bayne and Klug classification[360] (figure 1). Patients were included when at least in one of both limbs, there was a Type 0 RLD according to the modified Bayne and Klug classification, implying that cases with anomalies limited to hypoplastic thumbs (Type N) were excluded as these do not longitudinally affect the radial axis of development. Hand anomalies that co-occurred with RLD were classified according to the Oberg, Manske, and Tonkin classification for upper limb anomalies[21]. Associated anomalies were registered and categorized in 12 anatomical groups based upon our published registry system and the CulaPhen methodology: Cardiac, Respiratory, Respiratory, Digestive, Urogenital, Nervous system, Vertebral column, Musculoskeletal, Head/Neck, Lower limb, Skin, and Others[8,25].

Figure 1: Modified Bayne and Klug Classification

Type	N*	0*	1	2	3	4	5
							
Thumb	Hypoplastic or absent	Hypoplastic or absent	Hypoplastic or absent	Hypoplastic or absent	Hypoplastic or absent	Hypoplastic or absent	Hypoplastic or absent
Carpus	Normal	Absence, hypoplasia or coalition	Absence, hypoplasia or coalition	Absence, hypoplasia or coalition	Absence, hypoplasia or coalition	Absence, hypoplasia or coalition	Absence, hypoplasia or coalition
Distal Radius	Normal	Normal	>2mm shorter than ulna	Hypoplastic	Physis absent	Absent	Absent
Proximal radius	Normal	Normal, radiulnar synostosis, congenital radial head dislocation	Normal, radiulnar synostosis, congenital radial head dislocation	Hypoplasia	Variable hypoplasia	Absent	Absent
Humerus	Normal	Normal	Normal	Normal	Normal	Normal	Absent proximal humerus
Dominant grade of thumb hypoplasia	Type 2, Hypoplasia thenar, unstable MCP joint, narrow 1st web		Type 3 a/b, Type 2 + hypoplasia of extrinisic musculature with or without stable CMC	Type 3 a/b, Type 2 + hypoplasia of extrinisic musculature with or without stable CMC	Type 5, Absent thumb	Type 5, Absent thumb	Type 5, Absent thumb

Both the syndrome diagnoses and molecular work-up were reviewed in the database of the department of clinical genetics. As the source population is a historical cohort, primarily genetic work-up varied from karyotype to diagnostic whole exome sequencing. Variants and structural chromosomal variants were classified into different categories: 1) pathogenic variants, 2) New likely pathogenic variants, 3) Variants of uncertain pathogenicity.

Patients and parents of patients with: 1) bilateral occurrence, 2) multiple congenital anomalies without a confirmed molecular diagnosis were contacted if possible and offered further analysis using next generation sequencing. If interested, patients were invited for further consultation, and written consent was obtained from the patient and their parents (MEC-2015-12). Patients with combined RLD and cardiac anomalies who had not been evaluated for variants in *TBX5* and/or *SALL4* were analyzed in a 2-step protocol: first, *TBX5* and *SALL4* were evaluated by singleton NGS sequencing or by direct sequencing of these two genes as a part of regular clinical care[366,368]. If no pathogenic variants were obtained in the first step, the second step was the analyses defined in the research protocol.

Next generation sequencing

Next generation sequencing was performed on two platforms: WGS by Complete Genomics (GC), CA, USA) and WES by Biomics (Rotterdam, the Netherlands). Platform choice was based upon availability of WGS slots. Prior to sequencing, the DNA quality of the samples was measured by PicoGreen (ThermoFischer Scientific, Waltham, MA, USA). High coverage (>80x) WGS was performed as described by Drmanac et al.[369]. Mapping and variant calling was subsequently performed as described by Carnevali et al.[370] Exomes were captured using the Agilent Sureselect CRE kit and prepped according to the Illumina TruSeq v3 protocol. Samples were subsequently sequenced using the Hiseq4000 PE 150bp + 9bp index. All samples were aligned against human reference genome build 19 (hg19). Annotation of the NGS data was performed using the in-house developed pipeline using Annovar, Public60, and 1000 genomes data[60,61,341]. Variants were filtered according to read quality and allele frequency in the reference databases (Wellderly, GoNL), depending on the suggested mode of inheritance[59,60]. Gene predictions were made based upon the CulaPhen methodology; the produced list of RLD related genes is available in the supplementary files[25]. Separate variant files were created for denovo variants, recessive variants, compound heterozygous variants, maternally and paternally segregated variants. Both CG and Biomics provide CNV analysis; CG also provides structural variation data for the WGS. CNV and structural variant data analysis by CG is based upon discordant mate-pair analysis. Biomics uses read depth analysis applying the ExomeDepth R. Variants of interest were

validated using Sanger sequencing. The polymerase chain reaction products were sequenced employing the Big Dye Terminator 3.1. The fragments were loaded on an Abi 3130 sequence analyzer, and genetic analysis was performed with SeqScape Software (v3.0).

RESULTS

Clinical evaluation

One hundred eighty-eight patients with RLD were reviewed, 120 males and 68 female patients, 118 patients with bilateral anomalies, 71 unilateral (34 left, 37 right side affected). The baseline characteristics, including the severity of RLD according to the modified Bayne and Klug classification and the presence of other congenital anomalies, are depicted in Table 1.

Table 1. Baseline characteristics RLD population

	Isolated RLD		Multiple Congenital anomalies	
n	50		139	
Male/Female	35 / 15		85 / 54	
Left/Right/Bilateral	14 / 11 / 25		20 / 26 / 93	
Pos. family history cong. anomalies	14,0%		15,8%	
Severity				
Type N	3	4,0%	24	10,3%
Type 0	33	44,0%	78	33,6%
Type 1	12	16,0%	20	8,6%
Type 2	5	6,7%	8	3,4%
Type 3	4	5,3%	17	7,3%
Type 4	18	24,0%	84	36,2%
Type 5	0	0,0%	1	0,4%
Other CULA	34,0%		42,4%	

In 139 out of 188 patients, at least one other congenital anomaly besides the upper limb anomalies was observed. There was no linear relation between the severity of the RLD and the prevalence of associated anomalies; the highest prevalence was observed in Type 0 RLD and Type 4 RLD, 72,3 and 81,8%, respectively.

Associated hand anomalies included a wide variety of anomalies, most of which were other disruptions of the radio-ulnar axis development: triphalangeal thumbs n=14, radio-ulnar synostosis n=10, radial polydactyly n=6, and ulnar deficiencies of hand / longitudinal deficiency n=4/7. Other anomalies include syndactyly n=21, pectoral muscle deficiency n=18, and scapular deficiency n=2. Patients who also presented with pectoral muscle deficiency had the highest prevalence of associated anomalies, 88,9%.

Table 2. RLD versus general CULA cohort

	RLD	CULA	RR	(CI)
n	189	682		
Sexe (M/F)	120/69	392/290	0.777	(0.557-1.084)
Family history	15,3%	30,5%	0.413	(0.269-0.634)***
Bilateral occurrence	62,4%	48,1%	1.794	(1.289-2.496)***

					Observed anomalies, clinical and differential diagnoses		
Associated anomalies	**RLD**	**CULA**	**RR**	**(CI)**	**Observed anomalies include:**	**Most frequent clinical diagnoses**	**Differential diagnosis**
Circulatory	31,2%	6,2%	6.916	(4.461-10.720)***	VSD (n=30), ASD (n=25), Tetralogy of Fallot (n=8)	Holt-Oram syndrome (n=17), VACTERL/VATER (n=18)	Duane Radial Ray syndrome, Goldenhar syndrome, Klinefelter, Okhiro syndrome
Respiratory tract	10,6%	5,3%	2.12	(1.198-3.764)**	Tracheo/Laryngomalacia (n=9)	VACTERL (n=11)	
Digestive tract	24,9%	3,7%	8.698	(5.182-14.601)***	Esophagal atresia (n=24), Anal atresia (n=10)	VACTERL (n=31)	Goldenhar, Fanconi Anemia, TAR syndrome, Trisomy 18
Urogenital tract	20,1%	5,4%	4.387	(2.698-7.133)***	Kidney malformations (n=16), Cryptorchidism (n=9)	VACTERL (n=13)	Holt-Oram, Goldenhar, Fanconi, TAR
Neurological	6,3%	10,9%	0.557	(0.296-1.049)	Tethered cord (n=2), Retardation (n=2), Facial paralysis	VACTERL (n=4)	Holt-Oram, Goldenhar
Vertebral anomalies	16,4%	4,5%	4.120	(2.432-4.981)***	Scoliosis (n=13), Hemivertebrae (n=3)	VACTERL (n=10)	Goldenhar, Phocomelia, Holt-Oram, Klinefelter, Okihiro
Musculoskeletal	14,8%	2,1%	8.298	(4.271-16.123)***	Pectus excavatum/carinatum (n=6), Abnormal shoulder formation (n=12)	Holt-Oram syndrome (n=4), VACTERL (n=7)	TAR syndrome, Poland syndrome*

					Observed anomalies, clinical and differential diagnoses		
Associated anomalies	**RLD**	**CULA**	**RR**	**(CI)**	**Observed anomalies include:**	**Most frequent clinical diagnoses**	**Differential diagnosis**
Head/neck	29,6%	19,4%	1.754	(1.217-2.528)**	Cleft lip/palate (n=8), Ear anomaly (7), craniosynostosis (n=6), hemifacial microsomia (n=4)	VACTERL n=11Goldenhar n=4, Fanconi n=2, Baller-Gerold syndrome n=1,	
Lower limb	11,6%	27,0%	0.357	(0.222-0.574)***	Clubfoot (n=5)	Ohiro (n=2), TAR (N=3), VACTERL (n=4)	
Dermatological	7,4%	6,0%	1.251	(0.667-2.347)	Thrombocytopenia (n=8)	TAR syndrome (n=7)	
Others	14,3%	1,8%	9.306	(4.615-18.764)***	Cafe au lait spots (n=4)	VACTERL (N=4),	Poland syndrome, Baller-Gerold, Okhiro syndrome

Compared to a general population of CULA, patients with an RLD have a higher risk of bilateral occurrence and associated anomalies. Table 2 gives a detailed description of the affected anatomical groups, including risk estimates observed anomalies, and corresponding diagnoses. In 78 patients, a clinical diagnosis of a syndrome/association was made; most frequently diagnosed were VACTERL association n=38, Holt-Oram syndrome n=17, TAR syndrome n=7, Goldenhar n=3, Fanconi anemia n=3. Clinical predictors of these syndromes were evaluated and are displayed in Table 3.

Table 3. Statistically significant clinical predictors of syndromes/association

Syndrome	Associated anomalies	Prevalence	Odd ratio	p-value
Goldenhar syndrome n=3	Urogenital anomalies	75,0%	OR 12,4 (1,255-122,891)	*
	Vertebral anomalies	75,0%	OR 16,8 (1,689-167,5)	*
	Head/Neck	100,0%	-	**
Thrombocytopenia absent radius n=7	Absent radius (bayne >=4)	100,0%	-	***
	Well-developed thumb	57,1%	120 (15,52-927)	***
	Lower limb involvement	42,9%	OR 6,4 (1.338-30.944)	*
Holt-Oram n= 17	Bilateral affected	88,2%	OR 5.02 (1.11-22.67)	*
	Triphalangeal thumb	23,5%	OR 4.96 (1.37-18.1)	*
	Pectoral muscle hypoplasia	41,2%	OR 10.24 (3.27-32.13)	***
	Cardiac anomalies	100,0%	-	***
VACTERL(-like) n= 38	Cardiac anomalies	46,2%	OR 2,44 (1.18-5.05)	*
	Respiratory tract	28,2%	OR 6.16 (2.33-16.2)	***
	Digestive tract	82,1%	OR 41.14 (15.5-109)	***
	Urogenital anomalies	35,9%	OR 2.8 (1.28-6.12)	*

* p<0.05, ** p<0.01, *** P<0.001

In this historical cohort, structural variations were investigated using a Karyogram, FISH essay, or an Array in 88 patients. A chromosome breakage test was performed in 26 cases. In 40 patients were evaluated for variants in *TBX5*, *SALL4* was evaluated in 37 patients, and SALL1 in 24 patients. Results from these analyses are displayed in Table 4.

Table 4. Diagnostic yield within the historical cohort

		Yield			Observed variants	
		Normal	VUS	Pathogenic	VUS	Pathogenic variants
Karyogram/FISH/ Array	n=88	88,6%	8,0%	3,4%	Multiple small CNV's	XXY, XXX, Trisomy 18
TBX5	n=40	82,5%	5,0%	10%	756-28G>T, non-specified unclassified variant	c.587C>A; c.835>T; c.510+2dupT, unspecified pathogenic variant
SALL4	n=37	89,2%	5,4%	5,4%	c.3152 C>T; 1056G>A	2630ins8; 496insC
SALL1	n=24	91,7%	8,3%	0,0%	c.416C>T; 2544A>G,	
Chromosome breakage test	n=27	92,6%	NA	7,4%		

NGS evaluation of RLD patients

From the population of RLD patients with multiple congenital anomalies, 26 patients with their parent(s) consented for further genetic evaluation using NGS. The phenotypes of these patients included isolated limb anomalies (n=9), Holt-Oram-like (n=8), VACTERL like (n=7), and two patients with RLD combined with urogenital anomalies. A detailed list of families and observed variants are presented in Tables 5 and 6.

Table 5a. Detailed description of included families for NGS analysis: Isolated RLD with/without other musculoskeletal anomalies

	Platform	Included family members, affected/unaffected	Upper Limb Phenotype	Associated anomalies
Family 1	WGS	Female Index, affected.	Bilateral RLD	none
		Father, unaffected, related to mother		
		Mother, unaffected, related to father		
Family 2	WGS	Female Index, affected.	Bilateral RLD	none
		Father, unaffected		
		Mother, unaffected		
Family 3	WGS	Male Index, affected.	Bilateral RLD	Mild developmental delay
		Father, unaffected		
		Mother, unaffected		
Family 4	WES	Female Index, affected.	Bilateral RLD, Absent thumbs (disproportional to hypoplasia radius), Synostosis metacarpal 3-4, Radial head dislocation	
		Father, unaffected		
		Mother, unaffected		
Family 5	WES	Male Index, affected.	Bilateral RLD, Absent thumbs (disproportional to hypoplasia radius)	Coxa anteverta
		Father, unaffected		
		Mother, unaffected		
Family 6	WES	Male Index, affected.	Unilateral RLD, Bi-phalangeal fifth finger, synostosis metacarpal 4-5	
		Father, unaffected		
		Mother, unaffected		
Family 7	WES	Male Index, affected.	Bilateral RLD, Radial polydactyly unilateral	
		Father, unaffected		
		Mother, unaffected		

	Platform	Included family members, affected/unaffected	Upper Limb Phenotype	Associated anomalies
Family 8	WES	Male Index, affected.	Bilateral RLD, Unilateral syndactyly dig 4-5	
		Father, unaffected		
		Mother, Diethylstilbestrol exposure in utero		
Family 9	WGS	Male Index, affected.	Unilateral RLD, ulnar polydactyly	Cleft uvula, scoliosis, 11 ribs
		Father, unaffected		
		Mother, unaffected		

Table 5b. Detailed description of included families for NGS analysis: RLD with Cardiac anomalies

	Platform	Included family members, affected/ unaffected	Upper Limb Phenotype	Cardiac anomalies	Others
Family 10	WGS	Female Index, affected.	Unilateral RLD	VSD, patent foramen ovale, patent ductus arteriosus	Hypertelorism, lateral entropion, trichiasis, mild behavioral disorder
		Father, unaffected			
		Mother, unaffected			
Family 11	WGS	Male Index, affected.	Bilateral RLD, Triphalangeal thumbs	VSD	
		Father, unaffected			
		Mother, unaffected			
Family 12	WGS	Male Index, affected.	Unilateral RLD	ASD	mild behavioral disorder
		Father, unaffected			
		Mother, affected*	Brachydactyly type E		Hypertelorism
Family 13	WES	Female Index, affected.	Bilateral RLD, ulnar polydactyly	VSD	Bilateral Pectoral muscle hypoplasia
		Father, unaffected			
		Mother, unaffected			
Family 14	WES	Male Index, affected.	Bilateral RLD	AVSD, VSD	
Family 15	WES	Male Index, affected.	Bilateral RLD	VSD	Morbus Hirschsprung
		Father, unaffected			
		Mother, unaffected			
Family 16	Diagnostic sequencing *TBX5*/ *SALL4*	Singleton	Bilateral RLD, triphalangeal thumbs	VSD	Bilateral pectoral muscle hypoplasia
Family 17	Diagnostic sequencing *TBX5*/ *SALL4*	Singleton	Bilateral RLD, Triphalangeal thumbs	VSD	Bilateral Pectoral muscle hypoplasia

Table 5c. Detailed description of included families for NGS analysis: RLD with Urogenital anomalies

	Platform	Included family members, affected/unaffected	Phenotype	Urogenital anomalies
Family 25	WGS	Male Index, affected.	Unilateral RLD	Mono-kidney, hypospadia, urethral diverticulum
		Father, unaffected		
		Mother, unaffected		
Family 26	WES	Female index, affected.	Bilateral RLD, triphalangeal thumbs, proximal radio-ulnar synostosis	Renal fusion

Table 5d. Detailed description of included families for NGS analysis: VACTERL-like RLD patients

	Platform	Included family members, affected/ unaffected	Upper Limb phenotype	Vertebrae	Anal	Cardiac	Tracheal/ Esophagal	Urogenital	Others
Family 18	WGS	Female Index, affected	Bilateral RLD			VSD	Esophageal atresia, Tracheomalacia		Tethered cord
		Father, unaffected							
		Mother, unaffected							
Family 19	WGS	Male Index, affected	Bilateral RLD			AVSD	Esophageal atresia	Vesico-urethral reflux	tethered cord, hearing loss
		Father, unaffected							
		Mother, unaffected							
Family 20	WES	Female Index, affected	Bilateral RLD	Vertebral / rib anomalies	Anal atresia		Esophageal atresia		Cleft lip/palate, Duodenal atresia
		Father, unaffected							
		Mother, unaffected							

	Platform	Included family members, affected/ unaffected	Upper Limb phenotype	Vertebrae	Anal	Cardiac	Tracheal/ Esophagal	Urogenital	Others
Family 21	WES	Male Index, affected	Unilateral RLD			ASD	Tracheomalacia, Esophageal atresia		
		Father, unaffected							
		Mother, unaffected							
Family 22	WGS	Female index, affected	Bilateral RLD	Block vertebrae		ASD	Tracheomalacia		Growth retardation
		Father, unaffected							
		Mother, affected	Hypoplastic thumbs						
		Grandmother, affected		Block vertebrae					
Family 23	WGS	Male Index, affected.	Unilateral RLD		Anal atresia, rectourethral fistula		VSD, Patent Ductus Arteriosus		
		Mother, unaffected							
Family 24	WES	Male Index, affected	Unilateral RLD	11 ribs			VSD	Monokidney, Hypospadia	hip luxation
		Father, unaffected							
		Mother, unaffected							

Table 6. Results of NGS analysis

Isolated RLD with/without other musculoskeletal anomalies			
	Platform	**Observed class**	**Observed variant**
Family 1	WGS	Unknown significance	Compound heterozygote *DOCK6*; c.3724G>A, p.Gly1242Ser; c.3913C>T, p.Arg1305Cys
Family 2	WGS	Unknown significance	*GLI2*; c.671C>T, p.Thr224Met
Family 3	WGS		
Family 4	WES		
Family 5	WES		
Family 6	WES		
Family 7	WES	Pathogenic variant, non-RLD gene	GDF6; c.746C>A, p.Ala249Glu
Family 8	WES		
Family 9	WGS	Unknown significance	*GLI2*; Paternally inherited; c.4628G>A, p.Arg1543His
RLD with Cardiac anomalies			
Family 10	WGS		
Family 11	WGS	Unknown significance	Compound heterozygote ERCC4; c.1135C>T, p.Pro379Ser and c.2624A>G, p.Glu875Gly
Family 12	WGS	Pathogenic variant, non-RLD gene	DVL1; c.1147C>T, p.Pro383Ser
Family 13	WES		
Family 14	WES	Pathogenic variant, RLD gene	*TBX5*; c.668C>T, p.Thr223Met
Family 15	WES		
Family 16	Diagnostic sequencing *TBX5*/*SALL4*	Pathogenic variant, RLD gene	*TBX5*; c.408C:G
Family 17	Diagnostic sequencing *TBX5*/*SALL4*	Pathogenic variant, RLD gene	*TBX5*; c.511-949+1967del3069ins748
VACTERL-like RLD patients			
	Platform		
Family 18	WGS		
Family 19	WGS		
Family 20	WES	Unknown significance	TWIST1; c.94G>A, p.Gly32Ser
Family 21	WES		
Family 22	WGS	Likely pathogenic variant, matched phenotype	*NOP2*; c.1354C>T, p.Arg452*
Family 23	WGS	Unknown significance	SALL1; c.-934C>T, p.?

Isolated RLD with/without other musculoskeletal anomalies			
	Platform	**Observed class**	**Observed variant**
Family 24	WES	Likely pathogenic variant, matched phenotype	*TBX6*; c.602C
RLD with Urogenital anomalies			
	Platform		
Family 25	WGS		
Family 26	WES	Pathogenic variant, RLD-gene	*SALL4*; c.2491C>T, p.Arg831*

RLD and cardiac anomalies

A total of 9 families were eligible for inclusion. In this group, 4 patients had a pathogenic variation, three were observed in *TBX5* (c.668C>T, c.408C>G, c.511-949_663+1967del3069ins748) and one in *DVL1* (c.1147C>T). Interestingly, subclinical phenotypes were identified in 2 ancestors of these two families (*TBX5* and *DVL1*). The mutation in *DVL1* is described to cause Robinow syndrome, typically presenting with brachydactyly of the hands[371]. After clinical evaluation, the mother indeed had a brachydactyly phenotype. Interestingly, the assumed affected axis of development is different for brachydactyly and RLD.

In family 11, we have identified compound heterozygous mutations in *ERCC4* of unknown significance (c.1135C>T and c.2624A>G)[372]. Although prior analysis did not reveal Fanconi anemia, café au lait spots were present. The c.1135C>T mutation has been described in mild forms of xeroderma pigmentosum for homozygous carriers. The c.2624A>G mutation has a high population frequency (GoNL 1,70%) and is not described to be pathogenic. However, we observed an abnormal mitomycin C test in experimental assays, suggestive for a repair deficit (non-published data). Noteworthy, the sample of RLD patients with cardiac anomalies included a group of phenotypic copies (Family 13, 16, and 17) that resulted in pathogenic variants *TBX5*, but others remained unsolved.

VACTERL association

A total of 7 patients were included that had at least three features that fit in the VACTERL association. In family 22, we found a class 4 stop gain variant in all three affected family members affecting *NOP2* (c.1354C>T, p.Arg452*). Although little is known about this gene in human disease, it is expressed in all affected anatomical sites in mouse models[373]. A second patient was identified through Decipher with a stop gain mutation in the same exon of the *NOP2* gene c.1687C>T, p.Arg 563*.

After consultation of the submitting clinician in Decipher, the overlapping features include malacia of larynx/trachea, growth retardation, and cardiac anomalies. In family 24, a de novo class 4 *TBX6* variant, c.602 C>T, was observed in a patient presenting with RLD, a VSD, monokidney, hypospadia, and rib hypoplasia. The role of *TBX6* in congenital vertebral malformations is well established[374-378]. Furthermore, CNV analysis of congenital anomalies of the kidney and urinary tract has identified *TBX6* as an important candidate gene[379]. Verbitsky et al., besides identifying the focus, also illustrated the dosage-dependent kidney and urinary phenotypes in a knockout mouse model. In family 23, a class 3 variant in the promotor of *SALL1* was observed (c.-934C>T, p.?). Unfortunately, consent for functional essays could not be obtained. Lastly, the class 2 variant c.94G>A was observed in *TWIST1* in family 20. The index patient presented with a VACTERL-like phenotype and a cleft lip. Although this variant has been described as pathogenic for Saethre-Chotzen syndrome, hardly any overlap with this syndrome is present. Furthermore, the original case with this mutation also had a 22p deletion[380]. Other cases with this mutation presented with Saethre Chotzen or isolated craniosynostosis[381]. Thus, we are not inclined to relate this variant to the VACTERL genotype.

RLD with/without additional musculoskeletal anomalies

A total of 9 patients with either bilateral isolated RLD or RLD with additional musculoskeletal features were included. The yield of sequencing was one known pathogenic variant in *GDF6*, c.746C>A in Family 7. In Family 1 we observed compound heterozygote variants in *DOCK6* (c.C3913T and c.G3724A). Although both variants are predicted to be tolerated, it is interesting to note that a second patient in our source population had compound heterozygote variants in *DOCK6*, a gene related to Adams-Oliver syndrome[382]. Phenotypes of these two patients were overlapping; however the patient in the source population did present with aplasia cutis, whereas the index patients of the included family did not have a skin phenotype. Lastly, two patients had a VUS in *GLI2*, c.C671T, and c.G4628A. The index patient of family 2 presented with isolated bilateral RLD, whereas the index patient of family 9 had RLD, bifid uvula, scoliosis, a missing floating rib, and postaxial polydactyly. Pathogenic mutations in *GLI2* can cause Culler Jones syndrome; key features being postaxial polydactyly and hypopituitarism[383]. Although our patient has no record of hormonal insufficiency, features are variably expressed within families, and lacking hormone deficiencies have been described.

RLD and urogenital anomalies

Two families were included based on a combined phenotype of RLD and urogenital anomalies. In family 26, we observed a pathogenic variant in *SALL4* (c.2491C>T).

DISCUSSION

This study provides clinical and genetic insights into a historical cohort of patients with Radial Longitudinal Deficiency. Selecting this cohort based on the RLD phenotype instead of based upon clinical or molecular syndrome diagnoses, we established that patients with RLD overall have a higher prevalence of associated congenital anomalies. Although these associated anomalies often fit a clinical diagnosis, a genetic diagnosis was not commonly found in our historical cohort. Due to the historical nature of the cohort, renewed genetic diagnostics was warranted to provide sound recommendations for diagnostics. In a representative group of 26 families with no genetic diagnosis, we applied next generation sequencing to study the yield in the RLD population. Besides known pathological variations in 4 patients (*TBX5, SALL4*), we found variations in genes not related to RLD prior to this study (*GDF6, DVL1*). Additionally, candidate genes for RLD syndromes were identified in 8 more families: *NOP2, TBX6, DOCK6, GLI2*. This study can be used for clinical referral of patients to a geneticist, for evaluation of RLD patients, counseling, and correlation of NGS findings.

Although RLD can present in different severities, the severity of limb development disruption has no linear relationship with the prevalence of other congenital anomalies. The prevalence of associated anomalies in patients with RLD varied from 50,0 to 81,8%, with the highest prevalence obtained in both the lowest and highest classes of severity. These findings are not concordant with those of Halverson et al., who did observe a linear relationship[365]. These conflicting statements could be due to the exclusion of isolated hypoplastic thumbs in our population. Although severity was not a predictive factor, triphalangeal thumbs and disproportionally developed thumbs were associated with Holt-Oram and TAR syndrome, respectively. These findings are concordant with those of Forman et al., who described the relationship between the severity of thumb hypoplasia and hypoplasia of the radius, which in most cases is directly correlated[384]. However, the thumbs that were hard to classify thus not concordant with the average range of thumb hypoplasia described in the Blauth classification[384,385], were associated with Holt-Oram syndrome, TAR syndrome, and Fanconi syndrome. It is thus essential to familiarize oneself with the clinical spectrum of RLD, as captured in this work (Fig 1 and 2).

Patients with RLD have an increased risk of other associated anomalies than a general population of patients with congenital upper limb anomalies (RR 3.7). Many of the associated anomalies can only be detected when actively investigated (Table 2). However, in case by case review, nearly all patients with a syndrome had multiple hand anomalies or congenital anomalies detectable by physical examination alone, e.g., anal atresia, cleft lip/palate, or hemifacial microsomia. These findings

support the value of clinical examination and underline its value in the counseling on the indication for further genetic evaluation. Bilateral occurrence and clinically detectable associated anomalies could be used as a first threshold for referral to a geneticist and diagnostic evaluation. Special consideration should be given to lower limb involvement and pectoral muscle hypoplasia as clinical predictors of TAR syndrome and Holt-Oram syndrome and thus should warrant additional diagnostics such as a blood count or an echocardiogram (Table 3). Lastly, physicians should be aware that contralateral thumb involvement could only be based upon thenar hypoplasia; thus, thorough hand and physical examination is necessary.

In our population study, the spectrum of observed clinical syndromes was limited to 9 syndromes. This spectrum is more extensive than presented in most other studies, but still a small sample of the 29 syndromes available in literature-based upon our CulaPhen methodology (supplements). The most prevalent syndromes were VACTERL, Holt-Oram, TAR, Fanconi, Goldenhar, and Duane radial ray syndrome. Based on these findings, the primary genetic evaluation can be limited to *TBX5* and *SALL4*. Additional to these, an array, blood count or chromosome breakage tests, and/or a Fanconi panel analysis should be considered upon indication. Unfortunately, due to the small sample of Fanconi and TAR patients, our cohort provides little evidence on the risk of these syndromes. For TAR syndrome, as discussed, the absent radius with moderately developed thumbs is a clinical predictor. For Fanconi syndrome, 2 out of 3 patients presented with hearing loss which only occurred in 2 other non-Fanconi patients.

The clinical utilization of NGS for patients with RLD is seldomly described but is a valuable addition to the suggested primary genetic evaluation. Many strategies can be chosen in the evaluation of NGS results. Reflecting on our experience, limited analysis using gene panels restricted to those genes with a known association to RLD would have disregarded the variants in *DVL1* and *GDF6*. Therefore, a more comprehensive CULA based panel would be advised. Alternatively, a patient-only analysis could be applied; in our experience, this resulted in clinically irrelevant paternally/maternally transmitted variants. Trio analysis has the benefit of additional filter options based on the observed inheritance pattern, but comes with multiple downsides as well; First would be incidental findings: in our study, these were limited to two sub-clinically affected parents and the *TWIST1* variant that we argue not to be related to the observed phenotype. The second drawback would be the subclinical variants of parents that can be disregarded based on the assumed inheritance pattern. The last disadvantage is identifying variants of unknown significance or predicted pathological variants in genes yet unrelated to human diseases. For these findings, databases such as Decipher, GoNL, 1000 genomes are of imminent importance to interpret the results. However, these results bound the limit of clinical counseling and scientific research and could provide little value for the patient / family in question. From a counseling

perspective, a 3-step approach might be most feasible: primary genetic evaluation as discussed, secondary evaluation using a panel/filter for class 4-5 variants in a broad CULA-based panel, and finally, open trio analyses. From a cost perspective, the first two steps could be combined into one singleton WES, although this technology might not be available at a competitive price in all clinics. Based upon broad availability in other clinics, a "genome first" approach towards the genetic evaluation of patients with multiple congenital anomalies has been advocated. In our opinion, the clinical evaluation of patients with RLD is essential to indicate additional diagnostics and genetic sequencing. Therefore, we do not advocate a genome-first approach in the general RLD population.

The candidate genes for RLD related syndromes *NOP2, TBX6, DOCK6*, and *GLI2* require further validation but underline the necessity of genome-wide sequencing to further elucidate the genetic substrate of the anomaly. For both *NOP2, TBX6*, and *GLI2*, their role in limb and or musculoskeletal development is established through mouse models. For *DOCK6*, it is established that transverse limb defects can occur, which are a distinct limb phenotype to RLD. However, considering that two different patients with compound heterozygous variations in the gene could be included in this study, and considering one of these patients did present with aplasia cutis, we consider *DOCK6* a candidate gene for RLD. All candidates underline the importance of genome-wide sequencing in patients with RLD to identify candidate genes and indicate the necessity to report these findings to match genotypes and phenotypes.

Our study has limitations, the most obvious being that we could not include all patients for renewed genetic evaluation. By including patients with bilateral occurrence and/or multiple congenital anomalies, we knowingly introduced indication bias. In our experience, patients who did not meet these criteria were less commonly referred to a geneticist and did not express the will to evaluate the nature of the anomaly in the primary clinical evaluation. Patients who did meet the inclusion criteria refused genetic evaluation for various reasons, including the desire to allow the patient to co-decide in the matter at an older age, religion, and the lack of relevance to the patients' current phase of life. In this study, we used both whole genome sequencing and whole exome sequencing. In the analysis, however, both were primarily analyzed for coding variants, meaning data was left unused and diagnoses unmade. An example can be found in triphalangeal thumbs, which can be caused by mutations in regulatory domains of Sonic Hedge Hog. Because of the known regulatory landscape of *SHH* in relation to TPT, we were recently able to identify an additional zone that regulates *SHH* expression[386]. Unfortunately, for RLD, this regulatory landscape is not yet established. Further research should focus on the phenotypic copies of, e.g., *TBX5* phenotypes that we could not diagnose using our current NGS approach regarding only coding variants.

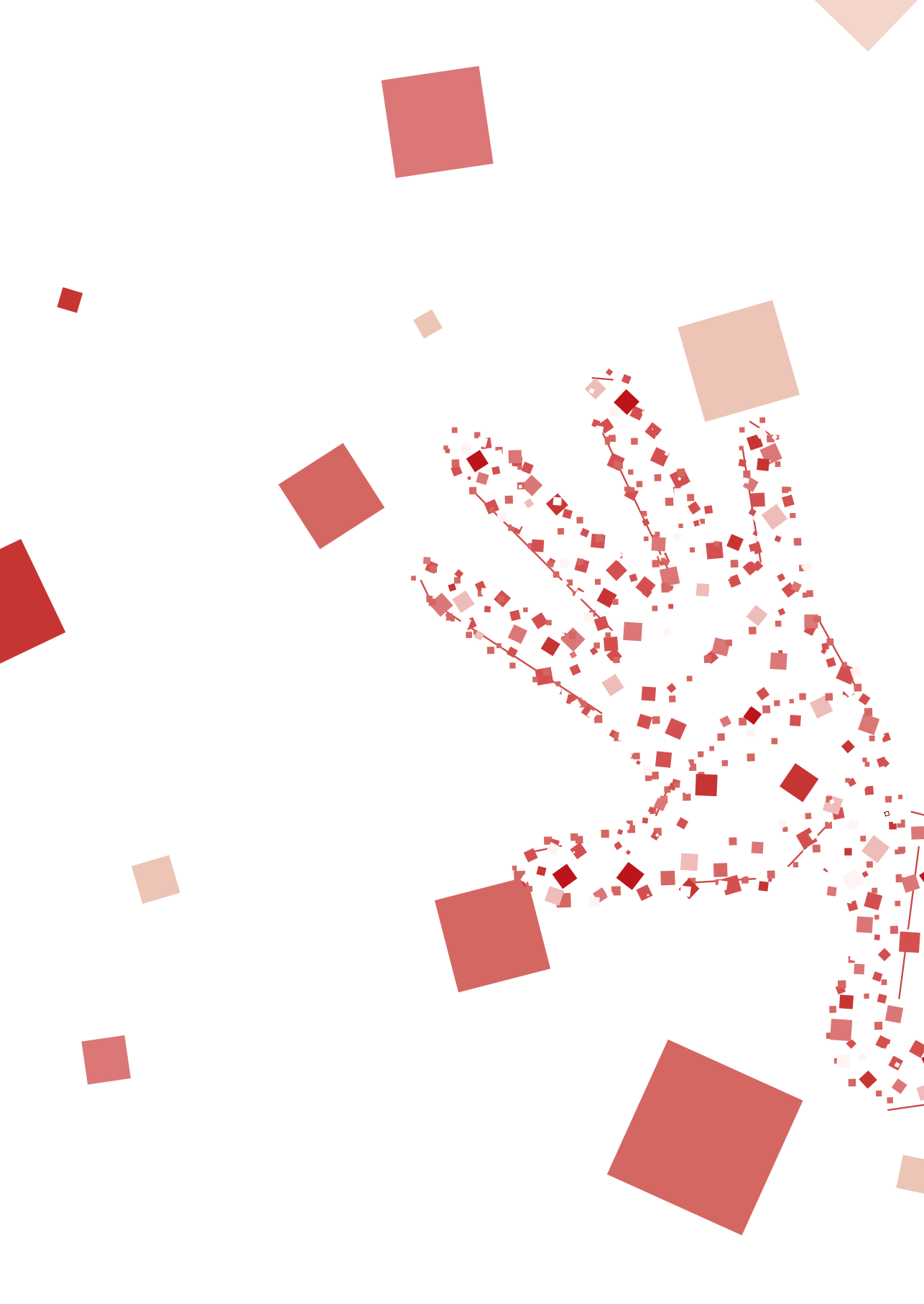
Figure 2. Variable thumb phenotypes in RLD. Typically, in type 3 or 4 RLD, floating or absent thumbs can be observed. However, in TAR syndrome, these thumbs are better developed. Although these thumbs are reasonably well developed, they do often lack extrinsic muscles; thus, they are not easily classified using the modified Blauth classification. Triphalangeal thumbs can also co-occur with RLD and can be an indicator of Holt-Oram syndrome.

Conclusion

Patients with RLD commonly have multiple congenital anomalies and are therefore often referred to a clinical geneticist. This study provides clinical features for referral and guides the evaluation and counseling of these patients and their families. Besides the commonly evaluated genes such as *TBX5* and *SALL4*, this study shows that genome-wide sequencing revealed pathogenic variants genes that are not commonly related to RLD; these variants would be missed in targeted diagnostics. Furthermore, four new candidate genes for RLD were established; *DOCK6*, *GLI2*, *TBX6*, and *NOP2*. Therefore, we advocate using NGS in RLD patients using a two or three-step approach to embed the differentiation between clinical and scientific outcomes.

SUPPLEMENTS

CulaPhen heatmap of all RLD syndromes. Related genes: *ATR, B2M, B3GALT6, BGN, BRCA2, BRIP1, CHD7, CHN1, COL11A1, COL25A1, DHCR7, DHODH, EIF4A3, ERCC4, ESCO2, EXT1, EXT2, FAM58A, FANCA, FANCB, FANCC, FANCD2, FANCE, FANCF, FANCG, FANCI, FANCL, FANCM, FGF10, FGFR1, FGFR2, FGFR3, FKBP10, FLNA, FLNB, GDF5, HOXA11, HOXD13, IHH, LMBR1, LRP4, MAD2L2, MAFB, MMP13, MMP9, MYH3, NIPBL, NPR2, P3H1, PALB2, PCNT, PDE4D, PITX1, PLXND1, PRKAR1A, RAD51, RAD51C, RBM10, RBM8A, RECQL4, REV3L, RPL26, RPS19, SALL4, SEMA3E, SF3B4, SHOX, SLX4, TBX3, TBX5, TGFB1, TNNI2, TNNT3, TPM2, TRIP11, UBE2T, WNT7A, XRCC2, ZBTB16, ZIC3*



10

General discussion

Patients with Congenital Upper Limb Anomalies (CULA) are commonly referred to (hand) surgeons to treat their anomalies. However, the role of a surgeon is more than just evaluating the need for conservative or surgical intervention. As a physician, especially when considering congenital anomalies, it is wise to have a broader scope and consider the pathophysiology of the observed anomalies. It is not uncommon that children are referred for the treatment of their CULA, which after thoughtful examination, turns out to be part of a rare syndrome or association that encompasses multiple associated anomalies. Missing these associated anomalies can have severe consequences for the child. To structure our examination and stratify the risk of such associated anomalies, it is helpful to structure our observations by using classification systems. For CULA, the recently introduced Oberg, Manske, and Tonkin (OMT) classification allows to describe the anomaly according to its nature, the extent of the anomaly, and the axis in which the anomaly is best described. Also, syndromes or associations can be registered, enabling epidemiological review of these CULA associated syndromes.

The OMT is a dynamic classification with several revisions/additions over the few years it has been introduced; all changes aimed to improve the classification based on knowledge gained in validation studies. This thesis also aims to add on to the knowledge that is already incorporated in the OMT classification and had three main goals:

- To improve the understanding of the relation between CULA and underlying pathology
- To evaluate the necessity of documenting all hand/foot anomalies in population studies
- To illustrate the value of in-depth phenotypic description for molecular diagnostics

PART 1: To improve the understanding of the relation between CULA and the underlying pathology.

The newly introduced OMT classification aimed to improve three features a classification for CULA should have, namely: 1) provide a classification that better fits the current concepts of the etiology, 2) allow combined documentation of all anomalies present in a single limb, 3) to cross-reference anomalies with syndromes. The last two aims of the new classification were not yet validated and were investigated in part 1 of this thesis. Tonkin, Ekblom, and Goldfarb have validated the OMT classification[2,55,72,387]. Their studies found that the OMT classification was an applicable and reasonably reproducible classification system. Combined documentation of limb anomalies was evaluated in Chapter 1. In 20.5% of the included patients, phenotypic information would have been lost if combined diagnoses had not been used. This data loss is best illustrated by the increased prevalence of Greig syndrome among patients with a combination of Radial Polydactyly, Ulnar Polydactyly, and Syndactyly (Chapter 6). Similarly, in our TPT population we would not have been able to confirm the presence of phenotypical anticipation if we had not combined the diagnoses observed in clinic (Chapter 4). From a pragmatic and an embryological point of view, it does not make sense to add complex triphalangeal thumb phenotypes to the classification. Pragmatically, this approach would have resulted in 47 new diagnoses when applied to every similar combination observed in our study. Embryologically, Chapter 4 shows that these complex phenotypes arise within a TPT population with the same ZRS mutation. Considering the fundamental principles behind the OMT classification, providing a classification that respects the current understanding of embryology, it would be incorrect to classify this as a separate entity; it is a subgroup of patients with the same genetic origin.

Combined diagnosing of multiple CULA in one patient might seem ideal; however, the effect on the inter-and intra-rater reliability is insufficiently studied in current literature. In the most extensive study on the reliability, it is stated that only cases with one diagnosis in 1 arm have been included for analysis[387]. This choice makes the analysis more straightforward but has the downside of not exploring the extent of multiple diagnoses we should classify. In our personal experience, registration of 4 or more diagnoses in one limb is hardly reproducible. However, the classification of up to 3 diagnoses is regularly required. Ideally, the inter- and intra-rater reliability should also be studied for multiple levels of combined diagnosis and weighted against the diagnostic value of each additional registered diagnosis. This study would have to involve large numbers of patients, raters, and preferably also a known embryological substrate for the anomaly for it to be valid. Thus, it is unlikely that an exact answer will be available at any timepoint soon. The pragmatic solution is to classify every additional anomaly that is not part of

the typical presentation, e.g., the hypoplastic aspect of a duplicated thumb does not require an additional registration of thumb hypoplasia, but radial polydactyly in radial longitudinal deficiency does. This approach has a major pitfall: the variety in personal or local experience with hand anomalies among OMT users, thus one's reference of a "common presentation." The OMT classification aims to provide a workable classification scheme for all specialists dealing with CULA; thus, experience by definition cannot be extrapolated between users.

The third aim of the OMT classification is to cross-reference anomalies with syndromes. For this purpose, we have developed the Culaphen methodology. With Culaphen, we provide an extensive cross-reference based on known associations in the Human Phenotype Ontology-project (HPO) dataset[104]. Comparing Culaphen directly to another HPO driven tool reveals that for patients with CULA, Culaphen is more precise. This large-scale cross-reference is, in our opinion, a step forward in creating a reference for CULA related syndromes. However, several additional steps could be taken to enhance availability and usability even further. First of all, CulaPhen would be more usable if developed for mobile devices on a stand-alone platform instead of using an external software package to power the tool. Second, extensive population studies to add data on the prevalence of syndromes for a specific CULA would help the clinician in the risk evaluation. Third, the prioritization of CulaPhen could further be improved if the prevalence of specific syndromes could be accurately estimated. The two last steps require large cohorts of patients to accurately determine the patients at risk compared to those with isolated CULA. Two initiatives help to achieve this future goal: 1) The development of a standardized health outcome measure set for CULA, including the OMT classification (ICHOM); second is the American initiative to develop a prospective congenital anomaly database (CoULD)[387-390]. For future cross reference, we encourage the participants to register associated anomalies, diagnosed syndromes, if the syndrome is genetically confirmed, and what genetic tests have been performed in general. These parameters will, in the end, provide a database based on which we can improve diagnostics, improve patient counseling and learn more about the common etiology of patients.

In chapters 1 and 2, we have discussed the benefits of documenting all anomalies and crosslinking them to the underlying syndromes. Chapter 3 illustrates the burden of inaccurate classification of a series of anomalies as Poland syndrome or sequence. In our systematic review, we reviewed 136 articles representing 627 patients with a broad range of anomalies that all described using the eponym "Poland syndrome." More importantly, when considering syndromes with pectoral deficiency, at least four other syndromes should be considered. We are not stating that in 38 out of 67 atypical cases, the actual diagnosis was missed. However, we want to emphasize that diagnosing these patients with "Poland syndrome" comes

with numeral assumptions regarding etiology, the risk for future children of the patient and his parents, and the risk of other anomalies in the patient. These factors are the critical difference between "Poland syndrome" and the other actual syndromes associated with pectoral muscle deficiency.

As a part of this thesis, chapter 3 also has another message which is less noticeable when reading the manuscript by itself. Over the last decades, the availability of data has grown tremendously. Often, we use a form of a search engine to structure the data for us. Subsequently, if we look for a reference to create a differential diagnosis for pectoral muscle deficiency, we will most likely end up with the most common syndrome for which pectoral muscle deficiency is obligate: Poland syndrome. However, as illustrated by our review, this is not always the correct one, and physicians might leave out many other options. Admittedly, these other options can be hard to find. For example, if you want to find an extensive population study of Holt-Oram syndrome in which pectoral muscle involvement is described, the most recent paper you would find was written in 1996[391]. This is another critical reason to use either CulaPhen or any other search engine built using an ontologically structured dataset, such as the data provided by the Human Phenotype project, the source data for CulaPhen.

PART 2: To evaluate the necessity of documenting all hand/foot anomalies in population studies

In part 2, we applied the gained knowledge in part 1 in our clinical practice using two highly prevalent anomalies in our population: patients with triphalangeal thumbs and patients with medial polydactyly of the foot primarily observed in Greig syndrome[392].

From clinical practice, we had learned that newer generations of the published triphalangeal thumb (TPT) population seemed to have increasingly complex hand anomalies. Using our registry, we could locate and revisit the TPT families and document all observed hand anomalies. We have shown that the severity of an inherited phenotype of affected patients in TPT families is not random but instead shows a pattern of increased severity over subsequent generations. The exact mechanism of the observed anticipation remains unclear. To our knowledge, this remains the only study on phenotypic anticipation in CULA. However, we have learned from personal communications that the suggestion of anticipation is present in several other CULA, non-TPT, cohorts. Again this observation and subsequent communications warrant us to develop a registry with enough flexibility to register combined diagnoses and family history.

In the light of current research within our research group, subclinical presentation of TPT phenotypes should be discussed as a potential cause of the observed anticipation[393]. If, for instance, we had only included severe cases because subclinical TPT's were regarded 'healthy' controls, we could have falsely interpreted a pattern of anticipation. However, the chance for this to have happened is minimal. First of all, we have revisited the families and have subsequently developed/adjusted the pedigrees accordingly. Second, in literature review, we found the same suggestion of anticipation[254,258,259]. There is evidence for deviance from normal mendelian dominant inheritance from both our data and the pedigrees in literature. Thus, there is no suggestion for missing subclinical cases. Moreover, by visiting the families, both affected and unaffected children of affected parents could be evaluated, thus without any referral bias.

By classifying all anomalies in our TPT population, we could quantify our clinical observation of anticipation. Our population study of medial polydactyly of the foot illustrates an inverse relation: by registering all anomalies, a clinical decision regarding diagnostics can be made. In general, there are three groups: isolated preaxial polydactyly of the foot, preaxial polydactyly accompanied by other hand and foot anomalies, and preaxial polydactyly accompanied by other malformations. The last two groups should be referred to a clinical geneticist. We provide a differential diagnosis based on a variant of the CulaPhen methodology. As discussed, this methodology does not provide discrimination in this differential diagnosis based on prevalence. More importantly, our population could be biased due to two separate types of referral bias. First of all, like the TPT population, there might be subclinical cases. The group of subclinical cases is likely to be bigger than the TPT population because patients might experience fewer physical limitations with medial polydactyly, and visually there is a large range of what can be regarded as a normal hallux in terms of broadness and stiffness. The second type of referral bias is introduced by the more severe syndromes that can co-occur with medial polydactyly. Due to these patients' physical and mental restraints based on the other malformations, they might never see a surgeon for a correction of their halluces.

The latter type of referral bias is one that will affect all cohort studies solely performed by (hand) surgeons and can have significant implications for our practices. For example, the improved quality of prenatal screening increasingly leads to prenatal referral of parents of unborn children to our outpatient clinic to counsel them based on our surgical experience with the anomaly. However, our surgical experience is limited because all patients were fit enough for referral/surgery at some point during their life. Thus, it is essential to realize that every patient that a hand surgeon enrolls in our registries is possibly contributing to a biased population of relatively healthy patients. Contrary, mild CULA might not be

recognized or are managed by non-specialized centers, thus introducing a bias that overestimates the complex anomalies. Therefore, preferably data collected by all different specialties dealing with congenital anomalies; e.g., surgeons, pediatricians, geneticists. To prevent a center-specific bias, introduced either by their sub-specialty or by population differences, preferably data of different institutions should be combined. Due to privacy safety considerations, it is nearly impossible to collect all data of 1 person under one identifier for this patient. Presumably, the best we can achieve is to pursue institution-wide inclusion in CULA registries.

PART 3 To illustrate the value of in-depth phenotypic description for molecular diagnostics.

In Part 1, our explorative studies started the cross-reference based on the OMT classification using a cohort of our clinic and using the HPO database. In Part 2, we have applied this knowledge using our population studies. In Part 3 of this thesis, we illustrated the relation of the observed phenotypes to molecular genetics. This part of the thesis focuses on better discrimination of phenotypes related to genotypes in GLI3 patients, identifying a new mutation in the preZRS region that causes complex hand phenotypes, and exploring the phenotype-based analysis of NGS in radial longitudinal deficiency.

In chapter 6, we have found that there are two distinct GLI3 phenotypes: a preaxial and a postaxial phenotype. We also showed that these phenotypes are significantly associated with different mutation types and locations within the gene. The effect of the different mutations in this report remains hypothetical, although other groups have conducted functional studies in the past for some of the mutations. The group of mutations that is best understood is probably the group with proven haploinsufficiency. Building upon this knowledge, we hypothesize that patients with a mutation affecting the activator region likely still have a functional repressor, leading to relative overproduction of the repressor and subsequently a postaxial phenotype. The suggested relation is relevant because we also found a relation between corpus callosum agenesis and mutation location. Although the data does not allow direct comparison of hand phenotype to corpus callosum agenesis, it is suggestive that patients with a postaxial phenotype have a greater risk for corpus callosum agenesis. This outcome of our analysis we believe is the ultimate example that classification of individual phenotypes is essential. Not just for accuracy but also to fulfill the 1st goal of the OMT classification: To provide a classification of CULA that relates to our increased and understanding of the etiology of anomalies and relate this understanding to determining which axis of development and differentiation is primarily involved. With our work,

we have illustrated that the etiology of preaxial vs. postaxial phenotypes are different; therefore, the way we classify these anomalies should reflect this. As demonstrated in chapter 1, the most efficient way of doing this is by combined classification of the diagnoses instead of an umbrella term such as synpolydactyly or pre- vs. postaxial polydactyly phenotypes: the first strategy lacks differentiation with, e.g., HOXD13 related phenotypes, whereas the latter terms require a level of knowledge one cannot assume every professional dealing with CULA to have.

Chapter 7 reports a Dutch/Australian family with triphalangeal thumb–polysyndactyly syndrome with a point mutation in a conserved noncoding regulatory region in the genome, a region adjacent to the Zone of Polarizing Activity Regulatory Sequence (ZRS), named the preZRS (pZRS). This point mutation was identified after several abnormalities known to cause a TPT-PS phenotype were ruled out, including point mutations, copy number variations (CNVs), and chromosomal rearrangements of the ZRS as well as pathogenic variants in other genes. We discovered a novel mutation in the pZRS that segregated with the disease and showed differential enhancer activity in transgenic mice. Our findings in chapter 7 yet again confirm the relation of the phenotype to the genetic substrate. Similar phenotypes to this family have been related to 2 groups of genetic variations: the last generation of patients with the 105 C>G mutation in the ZRS (chapter 4) and in cases with a microduplication encompassing the ZRS (and pZRS), which results in a spectrum from Haas-Type polydactyly to Laurin-Sandrow syndrome[334]. In the OMT classification, the observed hand anomalies in these groups would be classified as a triphalangeal thumb with additional other phenotypes (radial polydactyly, syndactyly, ulnar polydactyly) or ulnar dimelia (mirror hand), 1B2iv and 1B2v, respectively, thus indicating a close resemblance). Although alike, these phenotypes deserve to be two distinct entities. First of all, looking at Laurin-Sandrow syndrome, the hand anomalies tend to have an abnormal radio-ulnar orientation, i.e., a duplicated thumb/hallux in the center with digits five lateral. In contrast, complex triphalangeal thumb phenotypes remain to have a 'normal' radio-ulnar differentiation. Second, most triphalangeal phenotypes only affect the hands, whereas mirror-image polydactyly affects both hands and feet[277]. Interestingly, an inverse relationship between the complexity of the hand anomaly to the length of the microduplication has been observed. The exact functional difference between short and long microduplications is yet to be determined.

Chapter 8 revisits our local population of RLD patients; similar to the population studies in Part 2, this chapter provides indicators for referral and guides for clinical and genetic diagnostics. Prediction of genetic substrates using the CulaPhen methodology worked reasonably. However, it also revealed our limitations in knowledge of limb development: pathological variants known to be related to other CULA were observed in the RLD population, illustrating the interplay between the axis of development. In contrast to the predictable genetics of TPT and GLI3

phenotypes, in RLD patients, we found heterogeneity, and most importantly: in exact phenocopies, we were not able to diagnose both patients with a pathological variant nor a candidate gene. These findings humbled our confidence in the genotype-phenotype correlation of patients with CULA we developed during this thesis. They taught us four important lessons.

First of all, while theoretically NGS provides all data required for the genetic diagnoses in patients with CULA, in practice, this thesis illustrates that it is no substitute for genealogy and linkage analysis when locating and validating new genetic loci for Mendelian diseases. In line with this, a "genome-first" NGS approach in CULA patients seems overly simplistic for multiple reasons:

1. Not all patients require genetic evaluation, as illustrated in Chapters 6 and 9.
2. The phenotype is vital to determine the loci of interest, as illustrated by Chapters 3, 6, 7, 8, and 9.
3. The key phenotypic features that will identify the genetic loci of interest might not always be the hand anomaly, as illustrated in Chapter 9.

The above statements have implications for referral in clinic. With the introduction of NGS, one could suggest the NGS analysis as a prelude to referral to a clinical geneticist and only refer if clinically relevant findings are identified. We would argue against this approach because clinically relevant findings cannot be defined before evaluation. In close consultation with the clinical geneticists, a single gene or panel evaluation could be considered.

Second, the full potential of NGS for patients with CULA can only be unlocked when both genotypic and phenotypic data can be put together for evaluation. Especially those upper limb anomalies or syndromes that significantly affect the quality of life are rare, and the genetic substrate is heterogeneous. Genotypic data is commonly shared within databases such as Decipher, GeneMatcher, and Possum. Similarly, the international community dealing with CULA should strive for a reference database to connect patients with similar phenotypes. With our building experience in limb genetics, recently, we have experienced an increase of clinicians consulting us for the further genetic evaluation of, e.g., triphalangeal thumbs. However, the research precedes the publications that confirm that expertise to other centers; in other words: you will know what research your patient could have been included for once it is finished. Therefore, we should aim to build a platform for those physicians that deal with CULA to share phenotypes, genotypes, and research- or treatment protocols for rare hand anomalies and CULA syndromes.

The third lesson learned from Chapter 9 is that the axis of development we use to describe for normal development of the limb is merely a conceptualization; the influence of a given protein is not limited to a single axis. The interplay between axis is not a new concept, the presence of the "unspecified" class of malformations in the OMT classification is an indicator of that. The best example in this thesis is the family with a DVL1 variant causing brachydactyly in the mother and RLD in the son. However, these different phenotypes (brachydactyly vs. RLD) indicate that stringent filtering based upon the prioritization using CulaPhen will result in false-negative results. CulaPhen still has merit for the initial analysis: it is still more specific than other prioritization tools (Chapter 3). However, a staged approach with an additional open analysis after a panel analysis should be considered.

The last lesson originates from multiple chapters but was once more emphasized by the findings in Chapter 9: We are not aware of the full spectrum of phenotypes a genetic variant can produce. Please do consider sub-clinical phenotypes when analyzing NGS data. Not all hand phenotypes are observed by patients/parents, and not all hand phenotypes are observed by the clinician, even in a tertiary hand clinic.

Above and beyond: classification of CULA

The title of this thesis illustrates that there is more to classification than just the act of classifying itself. Assigning a class to the observed CULA should have implications for the professional classifying that anomaly, e.g., for referral, counseling, diagnostics, or the surgical treatment of the anomaly. Especially when concerning the OMT classification, which aims to serve users from different specialties with different roles in the treatment of patients with CULA, this implies that the introduction of a new classification warrants more validation than just validating usability by surgeons.

Besides introducing a renewed scheme, the transition from the IFSSH/Swanson classification to the OMT classification is a conversion that promotes splitting of phenotypes instead of lumping them together. Considering Part 1 of this thesis, this thesis supports this transition: it allows to define every needed nuance in the presentation of hand anomalies. However, part 2 and 3 also show the benefits of a lump strategy: e.g., simple vs. complex TPT phenotypes, preaxial vs. postaxial GLI3 phenotypes, isolated vs. syndromic medial polydactyly, etcetera. These findings suggest that when presented with the full spectrum of anomalies within a class, lumping can be feasible.

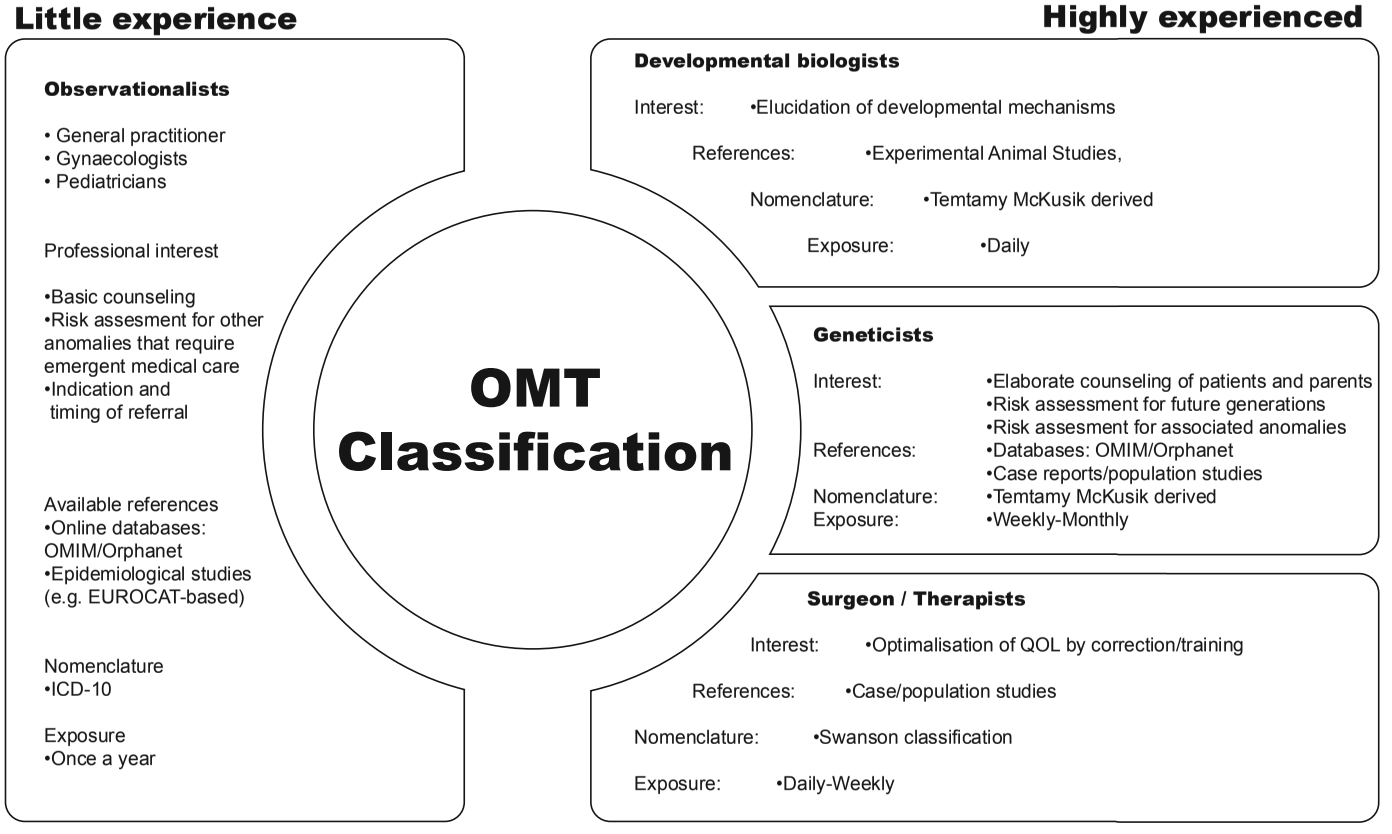
These "meaningful" lumps are meaningful for different reasons, e.g., developing a clinical work-up routine for medial polydactyly, decision-making for corpus callosum imaging, or guidance in genetic evaluation for TPT phenotypes. However, these lumps were primarily based on our experience with specific phenotypes that might not be relevant to other professionals or other centers. Our experience with TPT phenotypes is one of the key examples for this statement: professionals in our team will have the same interpretation of the term "complex TPT-phenotype," however, professionals from a different center will likely have a different representation of what this phenotype beholds.

Figure 1 again illustrates the difference in the role of the different medical specialists and scientists dealing with CULA, their interests, the references which they use, and the nomenclature they were trained with. These differences in background make that "meaningful" lumps differ per professional; they could be anomalies that should be referred to a specialist, or ZRS-mediated anomalies, or hand anomalies that warrant life-threatening conditions, or those conditions that need surgical correction. When developing a classification scheme and strategy with broad deployability, one should consider this difference in experience and interpretation of meaningful clusters: in communication, the recipient might be more important to consider than the sender of the message.

Therefore, aiming to go above and beyond the OMT classification means that we require an inclusive classification and concordant strategy that encompasses every phenotypic feature that might be relevant for one of the many different users of the OMT classification.

In my opinion, embryology, in the end, transcends the scheme developed; thus is "above" the classification. This thesis has illustrated that all anomalies have to be registered to appreciate the clinical presentation of CULA, its development over time, and the genetic substrate. The distal outcomes of the classification, or the "beyond" in the metaphor, are those actions clinicians take after classifying the anomaly. This thesis has shown that also for these outcomes, the presence of combinations of features can be decisive.

As a surgeon, the classification of some anomalies can seem redundant, as they might not always influence our surgical strategies. At these moments, we should remember that we do not only classify for ourselves, but also for the clinical geneticists we refer to, the epidemiologists who study the birth prevalence, and the embryologists who will try to interpret what we observe and translate this into a developmental model. However, in the end, you classify for your patient and those who will be referred to you, or your peers, in the future.


Little experience
Highly experienced
Observationalists
• General practitioner
• Gynaecologists
• Pediatricians
Professional interest
•Basic counseling
•Risk assesment for other anomalies that require emergent medical care
•Indication and timing of referral
Available references
•Online databases: OMIM/Orphanet
•Epidemiological studies (e.g. EUROCAT-based)
Nomenclature
•ICD-10
Exposure
•Once a year
OMT Classification
Developmental biologists
Interest: •Elucidation of developmental mechanisms
References: •Experimental Animal Studies,
Nomenclature: •Temtamy McKusik derived
Exposure: •Daily
Geneticists
Interest: •Elaborate counseling of patients and parents
•Risk assessment for future generations
•Risk assesment for associated anomalies
References: •Databases: OMIM/Orphanet
•Case reports/population studies
Nomenclature: •Temtamy McKusik derived
Exposure: •Weekly-Monthly
Surgeon / Therapists
Interest: •Optimalisation of QOL by correction/training
References: •Case/population studies
Nomenclature: •Swanson classification
Exposure: •Daily-Weekly

LIMITATIONS

Most of the limitations of this thesis have been discussed in the individual chapters. A few arise when discussing these studies as a part of the leading research questions of this thesis.

The relation of CULA to underlying pathology requires quantification to increase usability. In the current design of our studies and current formats of birth registries, this quantification is not readily available using the vocabulary of the OMT classification. To achieve this, preferably OMT terms should be adopted into the ICD-10 system to achieve acceptance at the most basic level of usage CULA terminology. Alternatively, ICD terms could also be linked to OMT terms like the CulaPhen methodology. However, when considering the conversion of ICD-10 based literature, the mismatch of terminology, the extend of case review necessary to provide accurate classification, and privacy considerations that you will meet when trying to do so will hamper any attempt to give OMT based birth prevalence using this method.

Although the necessity to document multiple anomalies in patients with CULA is illustrated by multiple studies conducted with multidisciplinary co-investigators, all studies were performed in a reference center for CULA. For primary physicians, the use of one single main diagnosis, or larger lump, might result in better referral guidelines: e.g., the children who present with complex TPT anomalies should be operated on early, whereas simple TPT can be corrected later. The usability of the complex OMT classification in primary care is therefore debatable.

When considering molecular diagnostics, we especially should consider the unsolved cases. The original TPT population now also shows complex TPT phenotypes, as shown in the pZRS patients, but we do not fully understand how this phenotype evolved. In the future, we might learn that different genetic or environmental factors influence the phenotype. Also, at the start of the analysis, we will not always realize what phenotypic features will overlap. In the family with the *NOP2* variants, we have found an overlapping phenotype in a second family, however, the other family did not have hand anomalies. Thus although precise phenotypic knowledge helps in the quest for finding a molecular diagnosis, one should also keep a broad enough view for patterns of associated anomalies.

Future Perspectives

Perhaps contradictory at first consideration, but the successful introduction of the OMT classification will result in many revisions of the classification, and at some point, we might need to disregard the structure of the classification altogether. Hopefully, the OMT classification will enable better structured data that is more readily available for direct comparison between clinics. From a congenital surgeon's perspective, this might enable predicting the development of a CULA over time, based on the follow-up of your peers. From a genetic point of view, this enables to match patients who might be distant relatives. This thesis and recent work by our research group illustrate that global migration is not something of this era but dates back decades. Therefore, the case treated by your peers in the USA might be distantly related to the patient who presented in Rotterdam. Improving this matching of patients is eminent in an era in which next generation sequencing is available as a diagnostic tool. However, these are not the main reasons why the OMT classification will be disregarded in the future.

The introduction of next generation sequencing has taught us a different lesson than I had anticipated. The overload of data obtained by NGS has again reminded us that most variations in the human genome are tolerated well and are non-disease causing. This has shifted the research interest into measuring and understanding biology rather than reading "code." For example, the ZRS was identified by linkage analysis, subsequent sequencing of the genome, and eventually functional evaluation using mouse models. In the future, we will appreciate that thumb anomalies are likely caused by a deficit in SHH-signaling, then we will measure the activity of all up-, and down-stream targets of SHH in cell lines of the patient, and that will result in a few coding or noncoding targets in the genome to sequence for identifying the causal variant. I am confident that this change in analysis strategy will eventually infiltrate all clinical medical work that will convert "Cancer" to a type of "DNA repair disorder," which better describes the nature of the pathology and what to expect in the future. For the classification of hand anomalies, this might result in abolishing "radio-ulnar axis disorders" and introduce "SHH dosage disorders."

As I just predicted the downfall of the OMT classification in a thesis dedicated to this one classification, I feel obliged to reflect on what aspects of the classification and this thesis are sustainable. In the coming years, the OMT classification should provide clinicians a framework to start registries that are the foundation for the transition described above. The first step to this goal was the acceptance of the OMT classification as the classification of choice by the IFSSH. However, perhaps more important is the inclusion of the OMT classification as one of the baseline characteristics to be registered when using the ICHOM dataset combined with

a detailed set of demographic factors. Databases, such as the CouLD-database, build upon these datasets, will collect prospective data on the treatment of the patients[387-390]. Hopefully, the multidisciplinary discussion started by forming the OMT discussion, and the research collaborations that have followed upon its introduction will prove to be omens of the true dedication to improve the treatment of CULA and data obtained by these outflows of the OMT classification.

11

Summary

Congenital Upper Limb Anomalies (CULA) arise in 1:500 births and behold a spectrum of isolated minor hand anomalies to invalidating syndromic anomalies. To structure this spectrum and to stratify the risk of associated anomalies, the OMT classification was introduced. This classification scheme aims: 1) to provide a classification that suits the current concepts of the pathophysiology, 2) to allow classification of all anomalies present in one limb, and 3) to allow cross-reference with syndromes. The last two aims of this new classification were further studied and tested in this thesis.

Part 1 - Classification of CULA Using the OMT Classification

In **chapter 2**, we applied the new classification strategy of combining diagnosis in a sample of our patient population. We have found that in 20,5% of cases, a combination was needed. Applying the combined diagnoses strategy prevented the introduction of 47 new entities and reduces the risk of over-stratification. Furthermore, within these 47 entities, multiple originated from a local TPT population and the high prevalence of Greig syndrome in our population. Since these are populations with a shared genetic cause of their hand anomalies, these entities should be classified as a variant rather than a separate entity. We conclude that the introduced classification strategy is indeed required to meet the aims of the OMT classification.

Once the classification strategy was established, in **chapter 3**, we explored the third aim of the OMT classification: to cross-reference hand anomalies to underlying syndromes. In order to reach this goal, we developed a tool that links currently available phenotypes in genetic literature to the phenotypes described in the OMT classification. For this purpose, we used the data available from the Human Phenotype Ontology (HPO) project. The product of our effort is called CulaPhen, which includes over 1400 syndromes and 1200 genes that can be related to congenital anomalies of the upper extremity. Comparing Culaphen directly to another HPO driven tool reveals that for patients with CULA, Culaphen is more precise in its prediction. Culaphen is an important first step in the cross-reference of syndromes to the OMT classification and underlines that a specific tool for hand anomalies, rather than a generic one for all birth defects.

Chapter 4 is focused on the burden of inaccurate or incomplete classification. Poland syndrome is commonly used as an eponym for any congenital absence of the pectoral muscle. Our systematic review shows the inaccurate use of the term, but more importantly, it also shows that within this selected study group 38 out of 67 atypical Poland cases have associated anomalies that could indicate the presence of one of the four genetic syndromes, identified using our CulaPhen

methodology, that include pectoral muscle deficiency. Chapter 4 illustrates that instead of using an eponym, we must keep classifying every anomaly with our preliminary syndrome diagnosis.

Part 2 - Distal outcomes of classification: population studies

In the previous chapters, we have focused on the OMT classification, its classification strategy, and its relation to syndromes. In Part 2, we focused on applying in-depth classification and cross-reference to syndromes to two of the most frequently presented anomalies in our outpatient clinic. In the end, it is not about the act of classification itself but about the distal outcomes that a classification system can provide. In **chapter 5**, we further studied patients with triphalangeal thumbs. In clinic, we have seen an increased prevalence of complex triphalangeal thumb phenotypes, including multiple additional thumbs, postaxial polydactyly, and syndactyly. Revisiting this population in the southern Netherlands, we have shown that these complex phenotypes do not occur randomly, but instead, there is evidence for phenotypic anticipation over generations: 71% of the latest generation had a more severe phenotype than their ancestor. This population study underlines the necessity of both combined diagnoses of the hand and the genetic diagnosis for epidemiological studies.

In **chapter 6**, we describe our population preaxial polydactyly of the foot. Using our database, using the same consideration of the OMT classification for the hand, and the CulaPhen methodology, we were able to establish three patient groups that warrant a different evaluation in clinic, namely: patients who have isolated preaxial polydactyly of the foot, patients with combined polydactyly of the hand and feet with no apparent other anomalies, and patients with other malformations. The first group has little risk of associated anomalies or a genetic substrate; thus, no further evaluation is warranted. The second group should be tested for mutations in GLI3. The last group should be referred to a clinical geneticist for further evaluation of underlying syndromes. This chapter illustrates a different type of distal outcome, namely its contribution to the formation of treatment guidelines based on our own experiences and the extensive data available in literature. A uniform classification and classification strategy can positively influence the availability of the latter.

Part 3 - Distal outcomes of classification: phenotype to genotype correlation

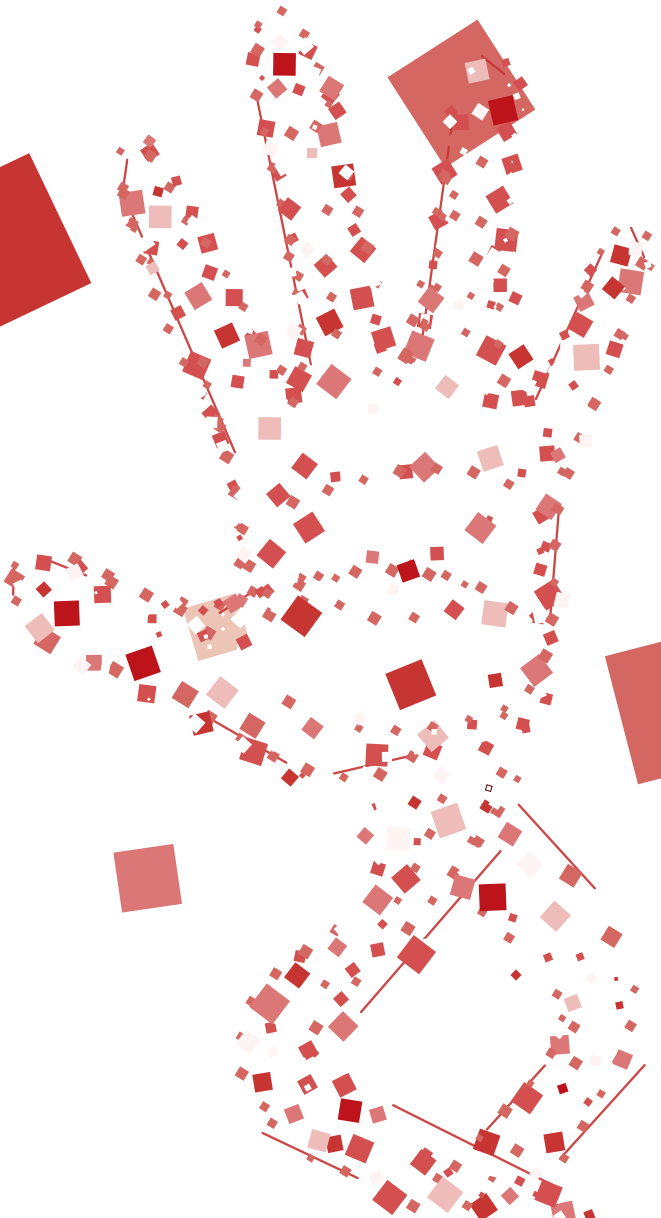
From **chapter 7** onward, we have focused on the influence of phenotype on the molecular diagnosis. Chapter 7 studies the GLI3 population in more depth. Using nearly 300 cases from either our population or literature, we found two distinct patterns of hand and foot anomalies, a group with primarily preaxial anomalies of the hand and feet and a group with primarily postaxial anomalies. These two distinct groups of phenotypes correlate with two groups of mutations in the GLI3 gene. Furthermore, patients with postaxial anomalies of the hand and feet have an increased risk of corpus callosum agenesis. Besides providing a renewed insight into the pathophysiology of Greig syndrome, this also provides clinical guidelines for intracranial diagnostics. Lastly, this also shows that combined diagnosis registration also has merit for nun surgical healthcare professionals dealing with these anomalies.

In **chapter 8**, we studied a second group of patients with TPT phenotypes. In contrast to the other population study, this study consisted of a homogenous group of complex TPT phenotypes without the typical ZRS mutation observed in the southern Netherlands. Using multiple distant family members, we identified a common mutation in a region near the known ZRS, named the preZRS. Using a mouse model, we confirmed that the mutation in the preZRS altered SHH signaling, a well-known pathway in hand development that is also responsible for the TPT phenotype in the ZRS population. As a part of this thesis, this chapter again illustrates the correlation of combined phenotypes, namely a complex TPT phenotype, with the underlying pathophysiology being impaired SHH signaling.

In **chapter 9**, we use next generation sequencing to try and find the genetic substrate of radial longitudinal deficiency patients. In this study, we have included several subgroups of patients with RLD in the hope to find similarly affected genes or pathways that influence hand development. On the contrary, we have found a wide variety of genetic variants, including: 1) known pathogenic variants for a syndrome known to cause RLD, 2) known pathogenic variant for a syndrome, not yet related to a CULA or associated with a different CULA, 3) New variants that by matching of patients could be linked to the observed phenotype. Furthermore, several (noncoding) variants were observed that warrant further functional analysis, not routinely practiced in diagnostics. Although this thesis focuses on the in-depth registration of hand phenotypes, these results implicated that stringent filtering based on phenotype will result in the loss of possibly pathogenic variants for genome-wide analysis. When studying the TPT or GLI3 mediated phenotypes, we have focused on disease primarily affecting hand and foot development. In RLD, especially in our selection with multiple congenital anomalies, we have to

consider that the underlying syndrome might primarily be of a different nature, such as cardiac. Moreover, subclinical phenotypes in parents should not be underestimated. It is very natural to perform a brief scan of parents when visiting the outpatient clinic with their child; however, we still encountered parents with a priorly unknown hand anomaly that matched the genetic inheritance. Combined, this warrants that genetic research is still a multidisciplinary effort, even more so in times of genome-wide sequencing.

As illustrated by the title of this thesis, there is more to the act of classification and to classification systems themselves: We have to consider what is above and beyond a classification. "Above" the classification resembles what is the true nature of the anomalies we observe in clinic and have to respect; what is the pathophysiology, and more importantly, does the framework we have created fit the mechanisms in nature? First of all, we have to conclude that the OMT classification strategy is necessary to classify all variations within one population and thus be embryologically valid. Secondly, studying these patterns does allow us to further elucidate these embryological processes and thus provides a distal outcome to the classification: the beyond. In this thesis, "the beyond" was illustrated by the formulation of clinical guidelines for referral to a geneticist, the demonstration of structural phenotypic anticipation, the increase of in-depth knowledge of GLI3-pathophysiology, and the identification of the preZRS locus with significant influence in SHH signaling. Finally, our sequence experiment has shown us that we must respect what is "Above" the classification: embryology, which is not limited to the hand and is essential to credit if we want better to understand developmental biology and its relevance to our patients.



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Nederlandse samenvatting

Aangeboren afwijkingen van de hand/arm afwijkingen komen voor bij 1:500 geboren kinderen en verschillen in ernst en aard van de afwijking. Daarnaast kan de afwijking op zichzelf staan, of kan voorkomen in het kader van meerdere aangeboren afwijkingen in het kader van een syndroom. Om deze verscheidenheid beter te beschrijven en om bijhorende risico's op bijvoorbeeld andere aangeboren afwijkingen te stratificeren, is recent de Oberg, Manske en Tonkin classificatie geïntroduceerd. Specifiek heeft dit classificatieschema de volgende doelen: 1) Het indelen van aangeboren afwijkingen van de bovenste extremiteiten op basis van de embryologische grondslag, 2) De mogelijkheid om meerdere afwijkingen binnen 1 ledemaat te beschrijven, 3) Een relatie te leggen tussen handafwijkingen en bekende syndromen die deze handafwijkingen kunnen veroorzaken. Deze laatste twee doelen werden in dit proefschrift verder bestudeerd en getoetst.

Deel 1 – Classificatie van afwijkingen van de hand/arm met de OMT-classificatie

In onze eerste studie (**hoofdstuk 2**), paste wij het concept van classificatie van meerdere afwijkingen toe op onze lokale populatie. Wij vonden dat in 20,5% van de kinderen, we inderdaad een combinatie van afwijkingen moesten classificeren om elke nuance in presentatie te registreren. Als gecombineerde registratie van diagnoses niet mogelijk was geweest, hadden 47 nieuwe entiteiten moeten introduceren met het risico van over-stratificatie. Binnen deze 47 combinaties verdienen de triphalangeale duimen de speciale aandacht. Deze duimen worden in verschillende combinaties gezien binnen een populatie waarvan we weten dat die genetisch homogeen zijn. Op basis van doel 1 van de OMT-classificatie zou dit dus niet correct zijn en concluderen wij dat gecombineerde diagnose registratie inderdaad een essentieel deel is van de classificatie strategie.

Nu de classificatie strategie is bevestigd, hebben we in **hoofdstuk 3** deze kennis gebruikt voor de identificatie van syndromen die voor kunnen komen in combinatie met een hand/arm afwijking. We hebben in dit hoofdstuk een tool ontwikkeld, genaamd CulaPhen,die hand/arm fenotypen en genotypen uit het Human Phenotype Ontology (HPO) project gebruikt en deze koppelt aan de handafwijkingen in de OMT-classificatie. CulaPhen bevalt in totaal 1400 syndromen en 1200 genen en kan beter voorspellen welke onderliggende syndromen waarschijnlijk zijn bij patiënten met hand/arm afwijkingen dan de beschikbare tools in de literatuur. Hiermee is de eerste belangrijke stap om syndromen te koppelen aan de classificatie gezet.

Om aan te geven dat classificatie niet zonder risico's is, hebben wij ook een studie gedaan naar de risico's van verkeerd verbruik van syndroom beschrijvingen. In **hoofdstuk 4** beschrijven wij dat het label "Poland Syndroom" vaak als synoniem gebruikt wordt voor afwezigheid van de pectoralis major spier. Dit is ten onrechte want er zijn tenminste nog 4 andere syndromen waarbij afwezigheid van deze spier kan voorkomen. In onze review laten wij zien dat 38 van de 67 patienten die niet het typische Poland syndroom hebben, wel afwijkingen hebben die passen bij die 4 andere syndromen. Dit pleit nogmaals voor het registreren van de individuele afwijkingen, eventueel aangevuld met een syndroom diagnose, in plaats van de klassieke classificatie methode volgens de IFSSH/Swanson classificatie.

Deel 2 – Uitkomsten van classificeren op populatieniveau

Waar we in deel 1 met name onderzoek hebben gedaan naar de OMT-classificatie, de relatie met syndromen en wat er fout kan gaan bij classificatie, gaat deel 2 over de waarde van classificatie voor klinische besluitvorming en onderzoek. Hiervoor hebben we twee populaties binnen ons ziekenhuis geselecteerd: patiënten met een verdubbeling van de grote teen en bijkomende hand/arm afwijkingen en patiënten met triphalangeale duimen.

In **hoofdstuk 5** hebben wij opnieuw onderzoek gedaan in een lokale populatie met patiënten met tirphalangeale duimen. In onze praktijk zagen we een toename van complexe hand afwijkingen binnen deze populatie, waar de voorouders met name een geïsoleerde triphalangeale duim hadden. In dit hoofdstuk concluderen wij dat er sprake is van fenotypische anticipatie; in 71% van de gevallen is de handafwijking ernstiger in de huidige generatie dan in de jongere generaties. Dit hoofdstuk laat dus zien dat uitgebreide registratie van de handafwijkingen in de kliniek leidt tot beter inzicht in het ziektebeeld.

Hoofdstuk 6 illustreert de waarde van uitgebreide classificatie voor klinische besluitvorming. Door de populatie kinderen met dubbele tenen uit onze kliniek te bestuderen, en de CulaPhen methodologie te gebruiken om gelieerde syndromen te identificeren hebben wij 3 verschillende groepen patiënten geïdentificeerd. Patiënten met geïsoleerde afwijkingen van de grote teen behoeven geen verdere verwijzing naar de klinisch geneticus. Patiënten met gecombineerde hand en voetafwijkingen, met name bestaand uit polydactylie en syndactylie zouden verwezen moeten worden voor onderzoek naar het *GLI3* gen. Als laatste is er een groep met uitgebreide geassocieerde afwijkingen die een risico op andere syndromen hebben. Deze patiënten moeten verwezen worden voor zowel klinisch als genetisch onderzoek. Om dit soort onderzoek te faciliteren, gebruiken we idealiter in de literatuur uniforme classificaties of nomenclatuur.

Deel 3 – Uitkomsten van classificatie op moleculair niveau

In het derde deel van dit proefschrift gaat de aandacht naar de relatie tussen het fenotype en de moleculaire genetische diagnose van de patiënt. In **hoofdstuk 7** bestuderen wij patiënten met een mutatie in het *GLI3* gen, dit is voor een belangrijk deel ook de populatie uit hoofdstuk 6. Door naast klinische patiënten ook patiënten uit de literatuur te gebruiken hadden wij bijna 300 patiënten beschikbaar, waarbinnen wij een patroon van hand en voetafwijkingen konden herkennen. In groep 1 zie je met name afwijkingen van de duimen en de grote tenen, in groep 2 zie je naast deze afwijkingen ook afwijkingen aan de pink/ kleine teen zijde van de hand en de voet. Of je in groep 1 of 2 zit is significant gecorreleerd met de locatie en het type mutatie wat in het *GLI3* gen gevonden is. Daarnaast vonden wij ook dat dezelfde mutaties die afwijkingen aan de pinken/ kleine tenen veroorzaken, ook een relatie hebben met de afwijkingen aan het corpus callosum die bij sommige patiënten is aangedaan. Deze uitkomsten illustreren dat de exacte locatie van de afwijkingen ons wat vertellen over de genetische oorzaak en sturing kunnen geven aan het klinisch onderzoek wat noodzakelijk is.

In het **achtste hoofdstuk** bestudeerden wij nogmaals een familie met triphalangeale duimen, alleen dit keer betrof dit een familie in Nederland en Australië die hele complexe afwijkingen had met meerdere extra duimen, pinken en syndactylie. Doordat we verre familieleden van elkaar konden includeren, konden we een klein deel van het genetische materiaal isoleren als meest waarschijnlijke oorzaak. Binnen dit stuk DNA vonden wij een mutatie in een nieuw regulatoir element, de preZRS, met nauwe relatie tot het regulatoire element waarin bij de bekende triphalangeale duimen de mutatie zich bevindt, de ZRS. Functioneel onderzoek heeft vervolgens bevestigd dat hetzelfde mechanisme is aangedaan bij deze mutatie, namelijk de regulatie van SHH-expressie in de handplaat. Het overlappende fenotype van de complexe afwijkingen in de bekende triphalangeale duimen populatie en deze nieuwe familie illustreert dat een vergelijkbaar klinisch beeld uiteindelijk ook een vergelijkbare pathofysiologie kent. Dus moeten we het klinische beeld zo betrouwbaar mogelijk vastleggen.

In **hoofdstuk 9** hebben we "next generation sequencing" gebruikt om patiënten met radial longitudinal deficiency (o.a. radius dysplasie) te onderzoeken met als doel een genetische diagnose te stellen die de afwijking veroorzaakt. Wij hadden verwacht voor de verschillende subgroepen binnen deze populatie vergelijkbare afwijkingen te vinden, echter vonden wij een breed genotypisch beeld. Dit beeld bestond uit: 1) mutaties in bekende genen die RLD veroorzaken, 2) mutaties in genen waarvan we wisten dat ze handafwijkingen konden veroorzaken, maar geen RLD, 3) mutaties in genen die we niet kennen in combinatie met handafwijkingen. Uit dit hoofdstuk hebben we meerdere belangrijke lessen geleerd. De eerste les is dat er binnen de

genetische analyse van handafwijkingen met NGS geen ruimte is voor een "genome-first" benadering. De tweede les is dat het delen van varianten gevonden met NGS voor loopt op het delen van de fenotypen, dit proefschrift laat zien dat we dat fenotype net zo uitgebreid moeten delen om deze resultaten te kunnen duiden. De derde les is dat de klassieke assen van ontwikkeling enkel een simplificatie zijn van de werkelijkheid om het begrijpelijk te maken, in de werkelijkheid lopen ze door elkaar en kunnen genen niet strikt tot 1 van de assen gerekend worden. Denken we wel zo strikt in de assen, dan verliezen we informatie uit de NGS-data. De laatste les is dat we door de invoering van NGS zullen leren dat we nu nog geen goed idee hebben van het werkelijke klinische spectrum van ziektebeelden.

Zoals de titel van dit proefschrift suggereert, is classificatie van een handafwijking meer dan het plaatsen van de afwijking binnen een klasse; we moeten ons afvragen op basis van wat dat klasse zo ontworpen is en dus wat het plaatsen van de patiënt binnen het hokje betekend voor de klinische behandeling en/of diagnostiek. De onderbouwing van een klasse is wat ik bedoel met de "Above"; de (patho)fysiologie van hand ontwikkeling. We moeten ons dus continu afvragen of ons ontwerp van de classificatie klopt bij de observaties in patiënten en de nieuwe inzichten vanuit moleculair biologen. De flexibiliteit van de OMT-classificatie geeft ruimte om de variabiliteit van het fenotype te beschrijven en gelijk dit te gebruiken voor verder (genetisch onderzoek). Dit verdere onderzoek is gelijk de "Beyond" uit de titel van dit proefschrift. Classificatie moet een doel hebben, wij illustreerden dat dit klinische beslisbomen kunnen zijn, epidemiologisch onderzoek en moleculair onderzoek. Ondanks de vele voorbeelden van een succesvol doel van de classificatie, heeft het laatste hoofdstuk ons ook laten zien dat "Above", of genetisch oorzaak altijd boven de classificatie staat, zich niet houdt aan de grenzen die wij stellen binnen de classificatie, of überhaupt niet altijd handafwijkingen zal produceren.

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LIST OF PUBLICATIONS

Re-innervation patterns by peptidergic Substance-P, non-peptidergic P2X3, and myelinated NF-200 nerve fibers in epidermis and dermis of rats with neuropathic pain.
Liron S. Duraku, Mehdi Hossaini, Barthold N. Schüttenhelm, Joan C. Holstege, **Martijn Baas**, Tom J.H. Ruigrok, Erik T. Walbeehm
Journal of Experimental Neurology 241 (2013) 13-24; PMID: 23219885

Innervation mapping of the hind paw of the rat using Evans Blue extravasation, Optical Surface Mapping and CASAM.
Shoista Kambiz; **Martijn Baas**; Liron S. Duraku; Anton L Kerver; Anton H.J. Koning; Erik Taco Walbeehm; Tom Johannes Hubertus Ruigrok.
J Neurosci Methods. 2014 May 30;229:15-27. doi:10.1016/j.jneumeth.2014.03.015. Epub 2014 Apr 8.

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Kambiz S, Duraku LS, **Baas M**, Nijhuis TH, Cosgun SG, Hovius SE, Ruigrok TJ, Walbeehm ET.
J Neurosurg. 2015 Feb 27:1-16.

Letter to the editor regarding: "Novel frame-shift mutations of GLI3 gene in non-syndromic
postaxial polydactyly patients"
Martijn Baas, Robert-Jan H Galjaard, Peter van der Spek, Steven ER Hovius, Christianne A van Nieuwenhoven
Clin Chim Acta. 2015 Apr 10. pii: S0009-8981(15)00187-4. doi: 10.1016/j.cca.2015.04.003.

A systematic review on the sensory reinnervation of free flaps for tongue reconstruction: Does improved sensibility imply functional benefits?
Martijn Baas, Liron S Duraku, Eveline ML Corten, Marc AM Mureau
J Plast Reconstr Aesthet Surg. 2015 Aug;68(8):1025-35. doi:10.1016/j.bjps.2015.04.020.

Identification of Associated Genes and Diseases in Patients With Congenital Upper-Limb Anomalies: A Novel Application of the OMT Classification.
Baas M, Stubbs AP, van Zessen DB, Galjaard RH, van der Spek PJ, Hovius SER, van Nieuwenhoven CA.
J Hand Surg Am. 2017 Jul;42(7):533-545.e4. doi: 10.1016/j.jhsa.2017.03.043.

Documenting Combined Congenital Upper Limb Anomalies Using the Oberg, Manske, and Tonkin Classification: Implications for Epidemiological Research and Outcome Comparisons.
Baas M, Zwanenburg PR, Hovius SER, van Nieuwenhoven CA.
J Hand Surg Am. 2018 Sep;43(9):869.e1-869.e11. doi: 10.1016/j.jhsa.2018.02.003. Epub 2018 Mar 21.

Intrafamilial variability of the triphalangeal thumb phenotype in a Dutch population: Evidence for phenotypic progression over generations?
Baas M, Potuijt JWP, Hovius SER, Hoogeboom AJM, Galjaard RH, van Nieuwenhoven CA.
Am J Med Genet A. 2017 Nov;173(11):2898-2905. doi: 10.1002/ajmg.a.38398. Epub 2017 Sep 10.

Preaxial polydactyly of the foot.
Burger EB, **Baas M,** Hovius SER, Hoogeboom AJM, van Nieuwenhoven CA.
Acta Orthop. 2018 Feb;89(1):113-118. doi: 10.1080/17453674.2017.1383097. Epub 2017 Sep 26. Review.

Controversies in Poland Syndrome: Alternative Diagnoses in Patients With Congenital Pectoral Muscle Deficiency.
Baas M, Burger EB, Sneiders D, Galjaard RH, Hovius SER, van Nieuwenhoven CA.
J Hand Surg Am. 2018 Feb;43(2):186.e1-186.e16.

A point mutation in the pre-ZRS disrupts sonic hedgehog expression in the limb bud and results in triphalangeal thumb-polysyndactyly syndrome.
Potuijt JWP, **Baas M**, Sukenik-Halevy R, Douben H, Nguyen P, Venter DJ, Gallagher R, Swagemakers SM, Hovius SER, van Nieuwenhoven CA, Galjaard RH, van der Spek PJ, Ahituv N, de Klein A.
Genet Med. 2018 Nov;20(11):1405-1413. doi: 10.1038/gim.2018.18. Epub 2018 Mar 15.

Variant type and position predict two distinct limb phenotypes in patients with GLI3-
mediated polydactyly syndromes.
Baas M, Burger EB, van den Ouweland AMW, Hovius SER, de Klein A, van Nieuwenhoven CA, Galjaard RJH.
J Med Genet. 2021 Jun;58(6):362-368. doi: 10.1136/jmedgenet-2020-106948. Epub 2020 Jun 26.

PHD PORTFOLIO

PhD Candidate:	M.Baas
Department:	Erasmus MC University Medical Center, Rotterdam Plastic and Reconstructive Surgery and Hand Surgery
PhD Period:	2014-2021
Promotor:	Prof. Dr. S.E.R. Hovius
Copromotors:	Dr. C.A. van Nieuwenhoven, Dr. R.J.H. Galjaard

1. PhD Training

Courses in methodology and statistics	**Year(s)**	**Workload (ECTS)**
NIHES Master of Genetic Epidemiology	2014-2016	70
General courses		
BROK course	2014	1
Erasmus MC research integrity	2016	0,3
English Biomedical Writing and Communication Course	2015	3
Medical skills		
Microsurgery training (4 hours/week)	2014-2017	300hrs
Tendon Reconstruction Course	2014-2017	0,7
Nerve Reconstruction Course	2014-2017	0,7
Local flap course	2014-2017	0,7
Oral Presentations		
Voorjaarsvergadering NVPC "Multidisciplinaire aanpak in de identificatie van genetische afwijkingen oorzakelijk voor handafwijkingen"	2015	1
Najaarsvergadering NVPC "Een systematische review over de sensibele reïnnervatie van vrije lappen bij tongreconstructies: wel of niet reinnerveren?"	2015	1

10th World Symposium on Congenital Malformations of the Hand and Upper Limb "Geographical differences in presentation of congenital upper limb anomalies in three specialized clinics"	2015	1
FESSH Milano - "The relation between the OMT classes and the indication for referral and research by a clinical geneticist"	2015	1
IFSSH, Buenos Aires - "The application of the Oberg Manske and Tonkin (OMT) classification in clinic; an evaluation of the classification strategy"	2016	1
IFSSH, Buenos Aires - "Identification of associated genes and diseases in patients with congenital upper limb anomalies; a novel application of the OMT classification"	2016	1
FESSH, Budapest: "Identification of associated genes and diseases in patients with congenital upper limb anomalies; a novel application of the OMT classification."	2017	0,3
FESSH, Budapest: "The application of the Oberg Manske and Tonkin classification in daily practice; an evaluation of the classification strategy"	2017	0,3
FESSH, Budapest: "Controversies in Poland syndrome: A systematic review on the presentation and definition of Poland syndrome in literature"	2017	1
4th European Symposium on Pediatric Hand Surgery and Rehabilitation, Paris: "Controversies in Poland syndrome: A systematic review on the presentation and definition of Poland syndrome in literature"	2017	0,3
4th European Symposium on Pediatric Hand Surgery and Rehabilitation, Paris: "The prevalence of Pectoralis Major hypoplasia in patients with Holt-Oram syndrome"	2017	1

Attended Seminars and workshops

24th Esser course 'Ins and outs of nose Surgery'	2014	0,3
NVPC, Voor en Najaar vergadering	2014-2017	1
10th World Symposium on Congenital Malformations of the Hand and Upper Limb	2015	1
FESSH Milano	2015	0,6
FESSH Budapest	2017	0,6
IFSSH Buenos Aires	2016	1
4th European Symposium on Pediatric Hand Surgery and Rehabilitation	2017	0,6

2. Teaching activities

Lecturing

3rd year medical school, master from head to hand: acute and chronic conditions of the hand	2015-2017	1

Skillslab

2nd year medical school: hand anatomy	2015-2017	2
3rd year medical school, master from head to hand: hand anatomy	2015-2017	2
Microsurgery course	2017	1

Supervision

Systematic review: Replantatie na vinger amputatie	2014	1
Master thesis: Pieter Zwanenburg	2015	3
Systematic review: CMC1 artrose	2015	1
Junior Med School: handphenotypen bij GLI3 mutaties	2015	2
Master thesis: Jaap Potuijt	2016	3
Project Medical delta: gene prediction in congenital upper limb anomalies	2016	2
Master thesis: Picard Nguyen	2017	1,5

3. Organising activity

10th World Symposium on Congenital Malformations of the Hand and Upper Limb	2015	8
25th Esser Course 'Oncoplastic Breast Surgery'	2017	4
All hands on deck, Farewell symposium Prof. Dr. S.E.R. Hovius	2017	4

CURRICULUM VITAE

Martijn Baas was born on July 7th, 1988, in Zwolle, the Netherlands. He grew up in the small village of Nunspeet. After six years of daily bicycle commute to Harderwijk, he graduated from the "RSG 't Slingerbos" (tweetalig VWO, NTG) with his profile paper on Quantum Physics under the supervision of the University of Utrecht.

After graduation, he started medical school at the Erasmus University in Rotterdam. During his electives, he was introduced to the intriguing discipline of Plastic and Reconstructive Surgery. Therefore, after his board year (S.S.R.-Rotterdam), he started his research career under the supervision of dr. E.T. Walbeehm, dr. L.S. Duraku and dr. S. Kambiz. His senior internship took place at the Department of Plastic and Reconstructive Surgery of the Erasmus MC (Prof. dr. S.E.R. Hovius), his elective internship took place at the Department of Plastic and Reconstructive Surgery of the Radboud University Medical Center (dr. E.T. Walbeehm / Prof. dr. D. Ulrich).

After his graduation in July 2014, he started working on his PhD-project on the classification and genetics of congenital upper limb anomalies (Prof. dr. S.E.R. Hovius, dr. C.A. van Nieuwenhoven, dr. R.J.H. Galjaard). During his PhD period, he was granted the opportunity to enroll in the Netherlands Institute for Health Sciences genetic epidemiology program, obtaining his second master's degree in the summer of 2016. After working as a resident (ANIOS) at the department, Martijn was accepted for the Plastic and Reconstructive Surgery residency program in the Erasmus MC in 2016. After his acceptance to the program, Martijn worked full time on his PhD project till December 2017.

In 2017, Martijn started his residency program in the general surgery department of the SFVG Franciscus (Dr. T.M.A.L. Klem). In September 2019, Martijn returned to the Erasmus MC and is expected to finish his residency program in 2023.