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GENETIC STUDIES OF CONGENITAL UPPER LIMB ANOMALIES

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Now faith is the assurance of things hoped for, the conviction of things not seen

- Hebrew 11:1

ABSTRACT

The overall aim of the work reported in this thesis is to improve epidemiological and genetic knowledge of congenital upper limb anomalies (CULA). To accomplish the specific aims of each paper several different methods and approaches have been used such as register studies, array comparative genome hybridization, Sanger sequencing, exome sequencing and functional studies using Lacz enhancer assay, Zebrafish morpholinos and computational transcription factor binding site prediction.

By screening all available medical records and X-rays retrospectively at all hospitals treating CULA in Stockholm county between 1997 and 2007 we identified 562 individuals with CULA resulting in an incidence of 21.5 per 10000 live births. In 99 of the 562 individuals (18%) there was a known occurrence of limb anomalies among relatives. One hundred and thirty of the 562 children (23%) had associated non-hand anomalies. A general table with all studied data that provides good counselling information about gender, laterality, associated anomalies and occurrence among relatives for each type of CULA was created.

The conserved Zone of polarizing Activity Regulating sequence (ZRS) restricts Sonic hedgehog expression to the posterior limb bud and thereby controls anterioposterior patterning in the upper limb. Sanger sequencing of the ZRS in a family with autosomal dominant inherited triphalangeal thumbs with an extra hypoplastic radial thumb revealed an insertion of 13 base pairs segregating with the phenotype. The insertion was predicted to add binding sites for several limb related transcription factors and in a Lac-Z enhancer assay, the insertion mimics the ectopic anterior expression of Sonic hedgehog in the limb bud previously reported to cause radial polydactyly.

A multicentre retrospective study was performed, based on six individuals with overlapping microdeletions of 17q22 including the *NOG* gene. Phenotypic and genotypic comparison between the six included individuals revealed a novel, previously not described 17q22 microdeletion syndrome with symptoms comprising common facial characteristics, multiple bone and joint problems including symphalangism, urogenital malformations and intellectual disability. In addition, some important differences were noted between the individuals, such as hypogonadotrophic hypogonadism and absence of uterus in one individual with a larger heterozygous deletion including *TRIM25*, also named EFP (estrogen-responsive finger protein) Mice carrying a loss of function mutation in one of the *Efp* genes have underdeveloped uterus suggesting that *Efp* could be involved in the normal estrogen-induced cell proliferation of uterus and the uterine swelling

Exome sequencing and Sanger sequencing in three unrelated families with the recently described condition X-linked recessive fusion of the fourth and fifth metacarpals, MF4 (OMIM309630), showed three novel variants in *FGF16*. In one of the families a truncating *FGF16* mutation also segregated with heart disease. The importance of *Fgf16* in mouse heart development has previously been shown. We performed zebrafish functional knockdown of *fgf16* and showed heart oedema besides the expected recently described reduction of fin size.

Taken together, this thesis provides information about incidence and comorbidity of CULA and adds knowledge about phenotype and genetic mechanisms underlying radial polydactyly, X-linked recessive MF4 and the novel 17q22 microdeletion syndrome.

SAMMANFATTNING AV RESULTAT

Medfödda missbildningar inom övre extremiteten varierar i allvarlighetsgrad från avsaknad av händer eller delar av händer till krokiga lillfingrar och fastlåsta böjsenor. Enligt den definition som använts i **studie I** föds 21.5 barn per 10000 med en missbildning inom övre extremiteten, vilket motsvarar 1.2 barn per vecka i Stockholms län med 2012 års födelsetal (28392, SCB). Målsättningen med denna avhandling är att öka kunskapen om epidemiologin kring och genetiska orsaker till missbildningar inom övre extremiteten.

Handen liksom ansiktet syns alltid och det är därför ofta lätt att upptäcka avvikelser i handens utveckling redan på det nyfödda barnet. Andra missbildningar kan vara svårare att upptäcka, särskilt de som drabbar inre organ.Förekomst av associerade missbildningar varierar mycket beroende på vilken typ av handmissbildning individen har. I **studie I** varierar denna siffra mellan 0 och 85% beroende på typ av handmissbildning (genomsnitt 23%). Arton procent av alla barn som ingår i studien har en släkting med övre extremitetsmissbildning. Hos personer med ej separerade fingrar (syndaktyli) var siffran ännu högre; 53% har en släkting med övre extremitetsmissbildning. Trettiosju procent av personerna med ej separerade fingrar har bilaterala övre extremitetsmissbildningar.

Vetskapen att vissa extremitetsmissbildningar förekommer familjärt och att de i många fall drabbar flera extremiteter och andra delar av kroppen gör det troligt att vissa missbildningar inom övre extremiteten är genetiskt orsakade.

I **studie II** har sekvensering av det reglerande fragmentet ZRS (Zone of polarizing activity Regulating Sequence) på kromosom 7 visat att en familj som ärver trefalangtummar (istället för de normala två falangerna) och underutvecklade extratummar har 13 baspar extra (insertion) i ZRS. ZRS begränsar normalt uttrycket av proteinet Sonic Hedgehog till lillfingersidan av handen. I en musmodell med familjens insertion kunde vi påvisa att insertionen kan driva ett patologiskt proteinuttryck på tumsidan av mustassen i överenstämmelse med den etablerade teorin att extratummar orsakas av felaktigt uttryck av proteinet Sonic Hedgehog på tumsidan av handen. Insertionen lägger till flera ställen på DNA-strängen som extremitetsrelaterade så kallade transkriptionsfaktorer kan binda till, vilket sannolikt är förklaringen till att den är sjukdomsorsakande.

Studie III beskriver sex individer som saknar en liten del (1,8 till 8,2 miljoner baspar) på ena kromosom 17. Individernas ansikten liknar varandra och de har i hög grad hörselnedsättning, urogenitala missbildningar och mental retardation samt ledproblem inkluderande underutvecklade/sammanväxta mellanleder i fingrarna. Det gemensamma deleterade området som upptäckts med array comparative genome hybridization innehåller en gen, *NOG*. Proteinet som uttrycks från *NOG* har en väldokumenterad roll i led och benutveckling i hela kroppen hos många djurarter inklusive människa. De gemensamma dragen hos patienterna i **studie III** utgör grund för identifiering av ett nytt syndrom, 17q22 mikrodeletionsyndrom, som är viktigt att ha i åtanke vid utredning

av individer med b.la. medfött underutvecklade/sammanväxta fingerleder (symfalangism).

I **studie IV** beskrivs tre familjer med X-bunden recessiv nedärvning av Metakarpale 4-5 fusion (MF4) och sjukdomsorsakande mutationer i genen *FGF16,* Fibroblast Growth Factor 16*,* som identifierats med exomsekvensering (alla gener i en individ sekvenseras) och sekvensering av enbart *FGF16.* MF4 innebär en medfödd underutveckling på lillfingersidan av handen som resulterar i att det fjärde och femte strålbenet växer ihop och det underutvecklade lillfingret pekar ut från handen, Handens funktion försämras och några individer med MF4 har amputerat lillfingret p.g.a att det bara är i vägen. Det könskromosombundna nedärvningsmönstret betyder att främst pojkar drabbas och att pojkar inte kan föra vidare anlaget till pojkar. I en av familjerna förekommer hjärtproblem i ökad utsträckning då flera individer drabbats av förmaksflimmer, hjärtinfarkt och hjärtförstoring. För att påvisa betydelsen av *FGF16* för arm och hjärtutveckling slog vi ut funktionen av *fgf16* i zebrafiskembryon. Zebrafiskarna fick halva normalstorleken på fenorna och hjärtödem. Tidigare musmodeller har påvisat att *Fgf16* har stor betydelse för hjärtutveckling hos möss. Under slutarbetet med studie IV där vi kommit fram till att *FGF16* var den sjukdomsorsakande genen bakom MF4 publicerade Jamsheer et al. för första gången en studie baserad på två individer med *FGF16* mutationer som orsak till MF4. Man konstaterade också att proteinet FGF16 uttrycks mellan tårna på möss. I övrigt är *FGF16´*s roll i armutvecklingen hittills outredd och studie IV samt studien av Jamsheer et al. stimulerar förhoppningsvis till vidare molekylära studier av hur *FGF16* bidrar till armutvecklingen.

Sammanfattningsvis klarlägger denna avhandling den genetiska orsaken till tre övre extremitetsmissbildningar: trefalangtummar med extratumme, underutveckling av lillfingersidan med sammanväxt av 4:e och 5:e strålbenet och ett nytt mikrodeletionssyndrom med b.la. underutvecklade/sammanväxta mellanleder i fingrarna (symfalangism). Vidare beskrivs frekvensen av associerade missbildningar, drabbad sida, förekomst hos anhöriga och kön hos individer med övre extremitetsmissbildningar. På grund av att en övre extremitetsmissbildning kan bli inkörsporten till en allvarlig diagnos, exempelvis Fanconis anemi, så är de fenotypiska samband vi beskriver i **studie I, III** och **IV** av stor betydelse för den utredande läkaren, oavsett specialitet. Incidensen av övre extremitetsmissbildningar i Stockholms län mellan 1997 och 2007 definieras till 21,5 per 10000 levande födda barn.

PUBLICATIONS

This thesis is based on the following papers, referred to by roman numerals in the text.

I. Anna Gerber Ekblom, **Tobias Laurell** and Marianne Arner.
Epidemiology of Congenital Upper Limb Anomalies in 562 Children Born in 1997 to 2007: A Total Population Study from Stockholm, Sweden.
The Journal of Hand Surgery (Am) 2010 35A:1742-1754

II. **Tobias Laurell**, Julia E VanderMeer, Aaron M Wenger, Giedre Grigelioniene, Agneta Nordenskjöld, Marianne Arner, Anna Gerber Ekblom, Gill Bejerano, Nadav Ahituv and Ann Nordgren.
A Novel 13 Base Pair Insertion in the Sonic Hedgehog ZRS Limb Enhancer (ZRS/LMBR1) Causes Preaxial Polydactyly with Triphalangeal Thumb.
Human Mutation 2012 33:1063–1066

III. **Tobias Laurell**, Johanna Lundin, Britt-Marie Anderlid, Jerome L Gorski, Giedre Grigelioniene, Samantha JL Knight, Ana CV Krepischi, Agneta Nordenskjöld, Susan M Price, Carla Rosenberg, Peter D Turnpenny, Angela M Vianna-Morgante and Ann Nordgren.
Molecular and clinical delineation of the 17q22 microdeletion phenotype.
European Journal of Human Genetics 2013 21:1085–1092.

IV. **Tobias Laurell**, Daniel Nilsson, Wolfgang Hofmeister, Anna Lindstrand, Nadav Ahituv, Julia VanderMeer, Anders Amilon, Göran Annerén, Marianne Arner, Maria Petterson, Nina Jäntti, Hans-Eric Rosberg, Peter A Cattini, Agneta Nordenskjöld, Outi Mäkitie, Giedre Grigelioniene and Ann Nordgren.
Identification of three novel *FGF16* mutations in X-linked recessive fusion of the fourth and fifth metacarpals and possible correlation with heart disease.
Submitted.

RELATED PUBLICATIONS

V. Anna Lindstrand, Giedre Grigelioniene, Daniel Nilsson, Maria Petterson, Wolfgang Hofmeister, Britt-Marie Anderlid, Sarina G. Kant, Claudia A L Ruivenkamp, Peter Gustavsson, Helena Valta, Stefan Geiberger, Alexandra Topa, Kristina Lagerstedt-Robinson, Fulya Taylan, Josephine Wincent, **Tobias Laurell**, Minna Pekkinen, Magnus Nordenskjöld, Outi Mäkitie and Ann Nordgren.
Different mutations in *PDE4D* associated with developmental disorders with mirror phenotypes
Journal of Medical Genetics 2014:51:45-54

VI. Anna Gerber Ekblom, **Tobias Laurell** and Marianne Arner.
Epidemiology of Congenital Upper Limb Anomalies in Stockholm, Sweden, 1997 to 2007: Application of the OMT Classification.
The Journal of Hand Surgery (Am) 2014 39A: 237-48

CONTENTS

ABBREVIATIONS

-/-	Both alleles are knocked out in the gene before $^{-/-}$
AER	Apical ectodermal ridge
Autpod	The region of the limb that will become wrist and fingers
EN1	Engrailed 1
FGF16	Fibroblast growth factor 16
GLI3	Gli family zinc finger 3
HAND2	Heart and neural crest derivatives expressed 2
HOX	Homeobox
ICD-10	International statistical classification of diseases and related health problems - Tenth revision
IFSSH	International Federation of Societies for Surgery of the Hand
LMBR1	Limb development membrane protein 1
LMX1B	Limb homeobox transcription factor 1 beta
MFCS1	Mammalian fish conserved sequence 1
NOG	Noggin
SHH	Sonic hedgehog
Stylopod	The region of the limb that will become humerus
TBX5	T-box 5
Wildtype	The phenotype or genotype of a species as it occurs predominantly in a natural population (the wild)
WNT7A	Wingless-type MMTV integration site, family member 7a
Zeugopod	The region of the limb that will become radius and ulna
ZPA	Zone of polarisation activity
ZRS	Zone of polarisation regulating sequence

Table 1 Capitalization and italization used for genes and proteins in this thesis.

Species	Gene Symbol	Protein symbol
Human	*FGF16*	FGF16
Mouse	*Fgf16*	FGF16
Zebrafish	*fgf16*	Fgf16

1 PERSONAL REFLECTION

The inspiration for this thesis comes from countless questions from parents and their children at the outpatient clinic, some of them being: How common is this? Why did my child get an upper limb anomaly? Is there anything else wrong with my child? The discomfort of not knowing and the frustration from the parents triggered me to start doing research on upper limb anomalies. The epidemiology study has given us most valuable support in our daily clinical work in investigating congenital upper limb anomalies. Nevertheless, classifications do not explain why anomalies occur. To explore genetic mechanisms of congenital upper limb anomalies this project continued at the Center of Molecular Medicine under the superb guidance of Ann Nordgren. Fortunately, with a great deal of underestimation of the complexity and workload it would take. Understanding the intricate methods used in genetic research sometimes exceeds the capacity of a paediatric hand surgeon's brain. But in the three genetic studies of this thesis it has been rewarding to be able to explain the cause of the upper limb anomalies to the families and also to know that future similar cases might have an explanation.

Since the time of completing a PhD thesis is a learning period, another part of this work has been to explore the different methods used in epidemiology and genetic research. A great effort has been made to select methods with different approaches to maximise learning opportunities.

Stockholm, 28 January 2014.

Tobias Laurell

2 INTRODUCTION

2.1 EARLY EXPERIMENTS

Chick and mouse studies have been fundamental to understand limb development since there are many similarities between the limbs in the different species; each has an upper arm, lower arm, wrist and digits. A lot of the current knowledge on upper limb development is based on four types of experimental procedures:
- Surgical manipulation of the chick limb bud where cells are moved in or removed from the growing limb bud. The chick limb bud is reached through a hole in the egg. An example of this method was when Saunders in 1947 showed that the apical ectodermal ridge, AER, produces signals that promote proximodistal outgrowth by removing cells from the AER which led to the formulation of the progress-zone model. [1]
- Cell-labeling studies to understand where cells move and which cells compromise various limb structures.
- Gene expression studies to visualize spatial and temporal patterns of the expression.
- Disruption or misexpression of target-genes to test gene function. Early studies tested gene expression by implanting beads of soaked morphogens into the chick limb bud. By doing so the Fibroblast growth factors were identified as crucial morphogens from the AER.[2] Knockout studies in mice of individual or combined genes is now routine in profiled limb development research laboratories.
-A fifth procedure is emerging: computer simulations in which all interacting genes can be taken into account in one model.[3]

2.2 EMBRYOLOGY OF THE LIMB BUD

Upper limb development in humans occurs in a span of four weeks, between weeks four and eight of gestation. It starts on day 27 of gestation when the lateral plate mesoderm cells proliferate and bulge out to form a symmetric limb bud. The limb bud consists of an ectodermal pocket that envelops the proliferating mesenchyme.[4] The ectoderm forms a ridge on top of the limb bud called the apical ectodermal ridge, AER. This specialized epithelial structure runs along the boundary of the dorsal and ventral ectoderm from the anterior to the posterior side of the distal limb bud (Figure 1). During limb bud growth, mesenchymal cells that become distanced from the AER will condensate and begin chondrogenesis. Mesenchymal cells that remain close to the AER will not differentiate in the beginning of limb growth and therefore the cartilage primordium of the limb skeleton will form in a proximal to distal sequence. That is, first humerus, then radius and ulna, then wrist and lastly the hand will form. A cartilage primordium of all upper limb bones has been formed by week 8 of gestation.

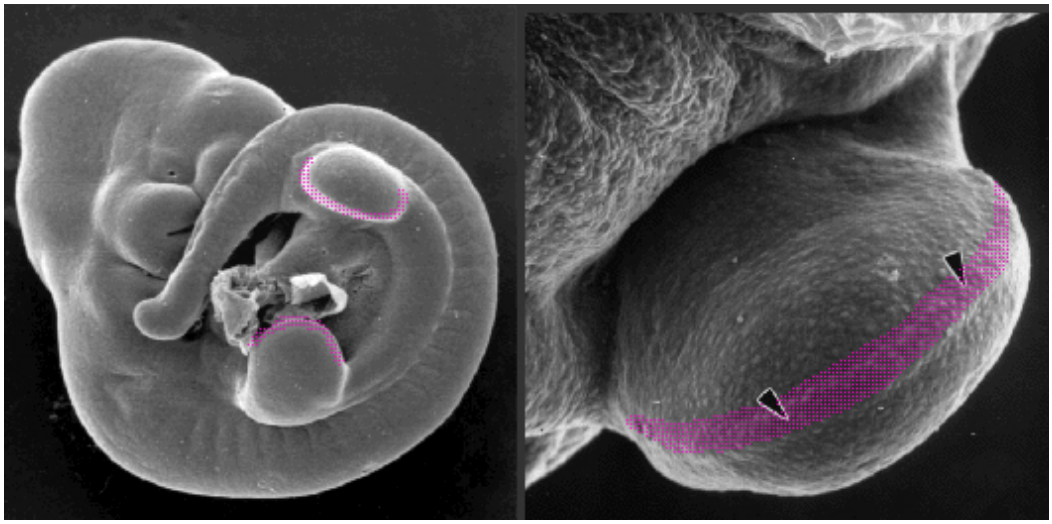

Figure 1. Apical ectodermal ridge on the limbud shown in purple. Scanning electron micrographs are from http://www.med.unc.edu/embryo_images/ and are reproduced with the permission of Kathleen K. Sulik, Ph.D.

The formation of the hand starts with a flattened paddle shape called the hand plate in day 32, figure 2. Mesenchyme cells in the hand plate condensate into five chondrogenic rays that will eventually become digits. At this stage the AER regress and the mesenchyme between the rays thin out. Eventually cells in the interdigital areas undergo apoptosis and five digits are formed by day 48 of gestation.

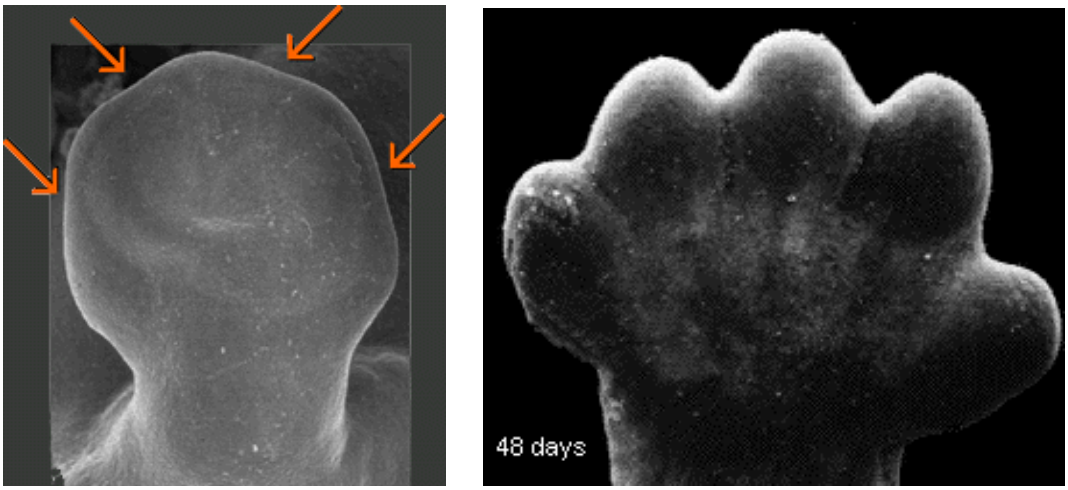

Figure 2 Red arrows indicate grooves at startsites for apoptosis in the handplate. Scanning electron micrographs are from http://www.med.unc.edu/embryo_images/ and are reproduced with the permission of Kathleen K. Sulik, Ph.D.

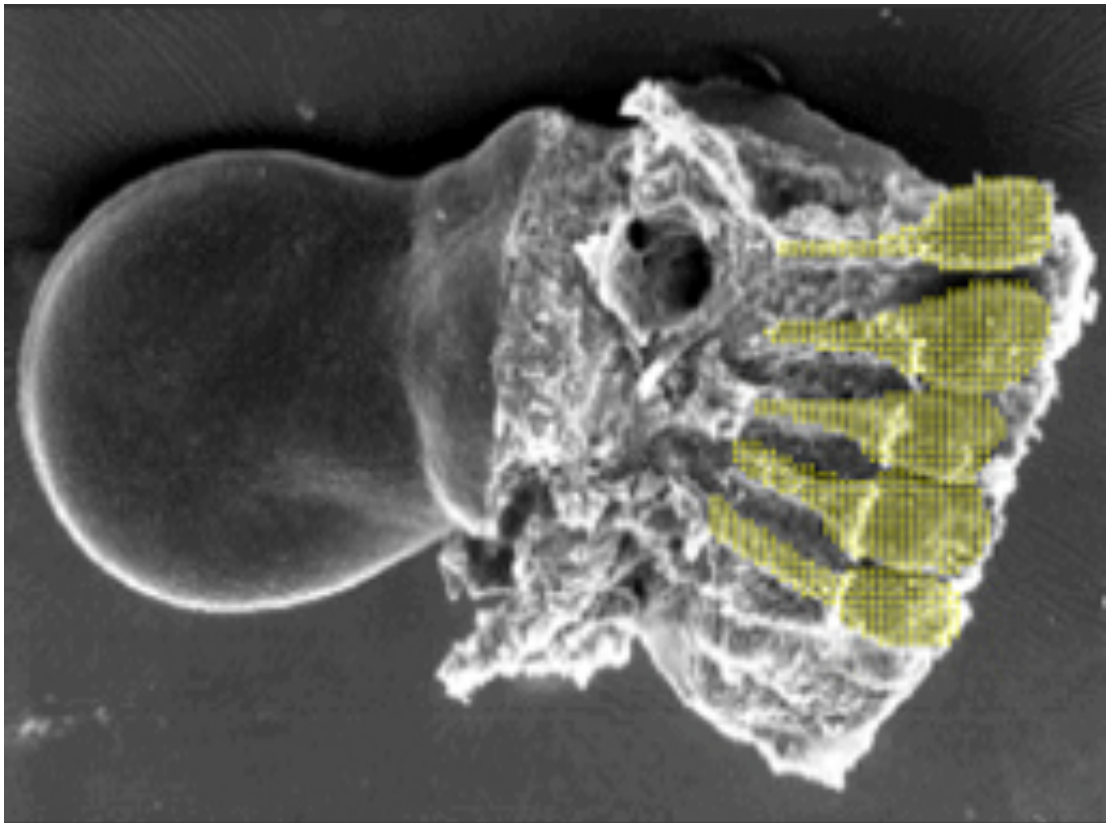

Figure 3 Yellow marking indicate brachial plexus growing into the developing limb bud at approximately day 33. Scanning electron micrograph from http://www.med.unc.edu/embryo_images/ and reproduced with the permission of Kathleen K. Sulik, Ph.D.

Most limb structures including bones, cartilage, tendons, ligaments and dermis are derived from the mesenchyme within the limb bud. Muscles, nerves and blood vessels grow into the limb from outside the limb bud. Muscle progenitor cells migrate from the somites to become dorsal and ventral muscle masses. These muscle masses then differentiate to individual muscles. Nerves grow into the muscles simultaneously (Figure 3).

2.3 GENETIC MECHANISMS OF UPPER LIMB DEVELOPMENT

2.3.1 Initiation and positioning of the limb bud

In all vertebrates the upper limb is formed at the level of the cervical-thoracic transition. Most likely *Hox* (Homeobox) genes define the position of the limb bud. *Hox* genes are expressed in a regional pattern, one after the other, in a variety of tissues along the whole cranio-caudal axis of the growing embryo.[5] The four clusters of *Hox* genes (A-D) are highly conserved among many species. In the upper limb field of the mesoderm, homologous *Hox* genes are expressed among many species indicating that there is a *Hox*-code for initiation and positioning of the limb bud.[6] *Tbx5*, T-box transcription factor 5 is selectively expressed early in the upper limb bud (and heart) development. In mice, *Hox* genes expressed in the lateral plate mesoderm regulate the forelimb-restricted expression of Tbx5 through a 361 basepair long regulatory sequence in intron 2 of Tbx5.[7] Thereby Hox genes, in interaction with Tbx5, facilitate to determine the position of the forelimb in a pre-limb bud stage.[7]

2.3.2 Patterning of the limb bud

Patterning is the process in which the morphologically symmetric limb bud gains asymmetry. Throughout the extensive history of limb development research, patterning has been described along three axes: *the proximodistal, the anteroposterior, and the dorsoventral*. However, as molecular knowledge rapidly increases it is now evident that these axes interact through common pathways.[4] The cross talk between cells is extensive and groups of cells in the limb bud are assigned a role as signaling centres. These signaling centres secrete morphogens, which act on neighbouring cells. Early experiments with removal, transferring and adding cells to and from different parts of the limb bud have helped to define these signaling centres.

2.3.2.1 Proximodistal patterning

Progress Zone model

Since removal of the Apical ectodermal ridge, AER, in early chicken wing buds leads to disruption of distal wing elements in the mature chicken it is clear that signals from the AER affect proximodistal outgrowth.[1,8] According to the progress zone model these signals keep the most distal mesenchyme cells of the limb bud in an undifferentiated and proliferative state. As the limb bud grows the undifferentiated cells just beneath the AER leave this progress zone and start differentiating. Cell identity, according to the progress-zone model, is thereby defined by the time mesenchyme cells spend in the progress-zone before they leave it. Thus the longer time cells spend in the progress zone under the influence of morphogens from the AER, the more distal their specificity will become.

Studies with fate mapping of chicken limb bud cells counters the idea of a progress zone in which cells, as the limb grows, specify what part of the limb they will develop into depending on how long time they spend in the progress zone.[9] Instead, fate

mapping shows that the early limb bud mesenchyme can be divided into smaller domains of cells, corresponding to the three different segments along the proximodistal axes. The mesenchyme cells could therefore be specified very early in limb development.

Fibroblast growth factor (FGF) signaling in the Apical ectodermal ridge (AER)

The significance of FGFs derived from the AER has been shown by the capacity of beads soaked in FGFs to restore limb outgrowth and patterning in chicken limb buds from which the AER has been removed.[2,10] In mice, several *Fgf* s are expressed in the AER: *Fgf*4, *Fgf*8, *Fgf*9, and *Fgf*17.[11] *Fgf*8 expression starts at the time of specification of the AER and continues as long as the AER remains.[12] Later *Fgf* 4, *Fgf* 9 and *Fgf*17 are expressed in the posterior AER and as the limb grows their expression expands in an anterior direction.[12,13] Conditional knockout studies in mice of individual *Fgf* s show that only *Fgf*8 is alone essential for limb bud development. These mice limbs get skeletal abnormalities along the whole proximodistal axis and are delayed with respect to Sonic Hedgehog expression.[12] Mice lacking expression of only one of *Fgf*4, *Fgf*9 and *Fgf*17 in the AER develop normal limbs.[13] When both *Fgf*4 and *Fgf*8 expression is absent in the AER, mice develop abnormal skeletal structures along the full extent of the proximodistal axis.[14] This contradicts the progress zone model in which conditional knockout of *Fgf*4 and *Fgf*8, from the AER, should disrupt distal development but not proximal. Defects observed following loss of *Fgf*8 function are also inconsistent with the progress-zone model. Instead, data support the hypothesis that *Fgf*8 is essential at very early stages to establish appropriately sized progenitor populations of all three limb segments.

The differentiation front model

To merge early grafting experiments and recent molecular data in a model of proximodistal limb development the differentiation front model was proposed.[4,15] In this model the early limb bud mesenchyme is undifferentiated, but as the mass of mesenchyme cells become larger, the most proximal cells will be out of range of FGF signaling and start differentiating to proximal structures (stylopod) under the influence of flank-derived signals. AER derived FGF signals keep the distal cells close to the AER in a proliferative undifferentiated state as the limb grows. The differentiation front is the border between progressively determined proximal cells and the specified but undifferentiated distal cells of the limb bud.

2.3.2.2 Anteroposterior patterning

French flag model

The zone of polarizing activity, ZPA, was identified as a signaling centre (small group of embryological cells that influence other cells) when grafts of mesenchyme cells where transferred from the posterior to the anterior side of the limb bud in chicken. By doing so, a mirror wing with bilateral posterior sides was created.[16] To explain the effects of the grafting experiments Wolpert formulated the “French flag model” in which a morphogen gradient spreads across the limb bud from the ZPA.[17] The three “wing digits” with individual morphogen gradient thresholds (colors in the French flag) will then specify as the mesenchyme cells are exposed to the right amount of

morphogen. In 1993 Riddle et al. identified sonic hedgehog, SHH, as the morphogen secreted from the ZPA.[18] Later, knockout studies of mouse embryos showed that *Shh*$^{-/-}$ mice only develop one rudimentary digit in the forelimb without anteroposterior identity.[19,20] Also, when transferring cells from the ZPA of *Shh*$^{-/-}$ mice to the anterior side of wild type chicken limb bud no mirror wing was created as was seen when doing the same between two wild type mice or wild type chicken.[19] The remaining digit in *Shh*$^{-/-}$ mice is most likely a thumb depending on the Sal-like 4 (SALL4), T-box 5 (TBX5) and HOX transcriptional regulators for development.[21,22]

Regulation of SHH in the limb bud

The limb specific *SHH* enhancer ZRS, (zone of polarizing activity regulating sequence), is located approximately 800kb upstreams of *SHH* in a highly conserved region in intron 5 of the limb region 1 gene, *LMBR1*.[23] Chromosome conformation capture assays have shown that the 800 bp region called MFCS1 (Mammalian fish conserved sequence 1) within the 1,7kb non-coding ZRS interacts physically with *Shh* by chromosomal looping and that the interactions are specific to cells in the ZPA.[24] Deletion of the MFCS (Mammalian fish conserved sequence) in mice results in the same limb phenotype as in *Shh*$^{-/-}$ mice.[25]

More than 20 different mutations (point mutations, duplications and one insertion) in the ZRS have been found to cause preaxial polydactyly in many animals including dogs, cats, chickens, and humans.[26] Experiments in mice and chicken with preaxial polydactyly due to ZRS mutations have shown that these mutations can alter regulatory function, causing ectopic anterior expression of SHH and increased posterior messenger RNA expression levels. Other ZRS mutations, not found in mouse models, have been tested in a mouse enhancer assay and shown to cause anterior expression of the reporter gene.[26] The mechanism that cause these distinct phenotypes and ectopic anterior expression is not fully clear but addition of transcription factor binding sites by the AGGAAGT motif have been suggested to contribute.[27] See the section of ”Discussion” for further details.

To position *Shh* expression in the posterior part of the limb bud, many factors are needed. Two of the key components are GLI3 and HAND2. HAND2 joins HOXD13 to form a larger protein complex that binds to ZRS in wild type mouse limb buds.[28] Timing of this HAND2 binding to ZRS is of great importance. When *Hand2* is conditionally inactivated early, *Shh* expression in mice limbs is abolished and the limb loses posterior structures (ulna and posterior digits) like in *Shh*$^{-/-}$ mice.[28] To restrict *Hand2* expression to the posterior limb bud *Gli3* is required.[28] Mice limbs without *Gli3* and *Hand2* function get shorter and does not obtain anterioposterior asymmetry and the autopod gets polydactolous.[4] Solitary *Gli3* inactivation in mice leads to polydactyly.[29]

Hox genes in anteroposterior patterning

Hox genes also control *Shh* expression in the posterior limb bud. Mice with loss of function mutations in the *Hoxa* or *Hoxd* clusters generate severe truncations along the proximodistal axis whereas gain of function mutants show more phenotype along the anteroposterior axes, eg polydactyly.[30] Hence, *Hox* genes seem to have affect on both proximodistal and anteroposterior patterning. Experimentally induced anterior expression of the normally posteriorly expressed *Hoxd11*, *Hoxd12* and *Hoxd13* genes

results in mirroring of *Shh* patterns and the extremity of mice.[31] Evidence that *Hox* genes act upstreams of *Shh* on anteroposterior patterning in the limb bud is the ozd chick mutant in which an anteroposterior *Hoxd11-Hoxd13* expression is established in the absence of SHH.[32]

2.3.2.3 Dorsoventral patterning

Regulation of dorsoventral patterning is less clear than along the other two axes, but some key components have been defined. LMX1B (Limb homeobox transcription factor 1 beta) is a homeodomain containing transcription factor that plays a role in dorsoventral axis formation since mice knockouts display distal ventral ventral morphology in bone, muscle and soft tissues.[33] It is expressed in a spatially and temporally restricted manner along the dorsoventral limb axis. *LMX1B* heterozygous truncating mutations in humans cause Nail Patella Syndrome, which is characterized by incomplete dorsalization of extremities, i.e., loss of the patella, deficient nails, and joint malformations.[34] *Lmx1b* is induced by *Wnt7a*, (Wingless-type MMTV integration site), which is expressed in the dorsal limb ectoderm.[35] In the absence of *Wnt7a*, the dorsal pattern of the distal structures (autopod) is not established and the limbs appear bi-ventral.

2.3.3 Tissue specific regulatory elements

The human genome contains only approximately 20,500 protein-coding genes.[36] The complexity of embryonic limb development and the low number of protein coding genes makes it intuitive that different cells need to use the same genes at different times and for different tasks during development. It is imperative for gene expression to be tightly regulated in space and time. In limb development there are good examples of cis-regulatory elements that complete this task. Cis-regulatory elements are regions in non-coding DNA that act on nearby genes by, for instance, enhancing or silencing a gene through physical interaction with the promoter. In limb development there are numerous reports of mutations in the enhancer ZRS (Zone of polarizing Activity Regulating Sequence). ZRS regulates *SHH* expression in the posterior part of the limb bud and mutations cause preaxial polydactyly of various severity, reviewed by Vandermeer et al.[26] Another example is microduplications of the *BMP2* (Bone morphogenetic protein 2) enhancer, which are known to cause Brachydactyly, type A2 (OMIM 112600).[37]

2.4 GENETICALLY CAUSED HUMAN CONGENITAL UPPER LIMB ANOMALIES

A large portion of present knowledge on limb development is based on animal models. The cause of many human congenital upper limb anomalies (CULA) is unknown. Nevertheless are there many inherited and sporadic genetic aberrations that indisputably lead to either isolated or syndromatic congenital upper limb anomalies. A filtered OMIM search for "limb" and "phenotype description, molecular basis known" generates 882 hits. A complete list of these is beyond the scope of this thesis, but there are some well-known examples worth mentioning:

Since the identification of the ZRS as a long-range enhancer for *Shh* more than 20 different point mutations, duplications and one insertion in the ZRS have been identified in humans with limb malformations.[26] These malformations represent a wide range of phenotypes. Large duplications including the ZRS and surrounding sequences cause complex Haastype polysyndactyly. Point mutations in the ZRS cause relatively less severe phenotypes including **radial polydactyly with or without triphalangeal thumbs**.

In **mosaic overgrowth**[38] and **macrodactyly**[39] causative somatic gain of function mutations in *PIK3CA* recently have been shown. In both conditions affected tissue has harbored mutations at p.His1047, which is a known hotspot for mutations in cancer.[40] PI3K-AKT inhibitors are currently under clinical trials for the treatment of various cancers[41] and one could speculate that also overgrowth conditions such as macrodactyly could be treated medically in the future.

There are five described syndromes associated with heterozygous *NOG* (Noggin) mutations: symphalangism in proximal interphalangeal joints (OMIM#185800), multiple synostoses syndrome (OMIM#186500), stapes ankylosis with broad thumb and toes (OMIM#184460), brachydactyly type B2 (OMIM#611377) and tarsal–carpal coalition syndrome(OMIM#186570). Thus far, a total of 36 human variations in *NOG* have been reported (https://grenada.lumc.nl/LOVD2/mendelian_genes/home.php,). All reported deletions of *NOG* on 17q22 have been associated with **symphalangism**. The collective term "NOG-related symphalangism spectrum disorder" has been suggested.[42]

In 1960 Holt and Oram reported a family with autosomal dominant inherited atrial septal defects and hypoplastic triphalangeal thumbs.[43] Subsequently **Holt-Oram syndrome** has been linked to mutations in the highly conserved T-box gene *TBX5* (OMIM#142900). Until 2004, 37 mutations in *TBX5* had been found in individuals with Holt-Oram syndrome.[44] Reported limb anomalies range from subtle carpal bone defects to triphalangeal thumbs, hypoplasia of thumbs and radius, digit aplasia and upper extremity phocomelia. The limb anomalies in individuals with Holt Oram Syndrome are typically bilateral and asymmetric, with the left side more severely affected than the right.

Ulnar Mammary Syndrome (OMIM#181450) is characterized by apocrine abnormalities of the thorax including breast defects and absence of axillary hair or

sweating in combination with bilateral reductions of the ulnar/posterior side of the limbs. Ulnar mammary syndrome is caused by autosomal dominantly inherited mutations in another highly conserved member of the T-box family, *TBX3*.

Synpolydactyly (OMIM#186000) is one of few human skeletal abnormalities resulting from *HOX* gene mutations. Typical synpolydactyly caused by mutations in *HOXD13*[45,46] occurs in all four limbs with cutaneous syndactyly between the third and fourth digit and a duplicated digit in between. The feet are similarly affected with cutaneous syndactyly and supernumerary toes, but more on the lateral side instead of central as in the hands.

Greig cephalopolysyndactyly syndrome (OMIM#175700) is caused by heterozygous mutations in the gene for one of the key transcription factors in limb patterning: *GLI3* (Gli family zinc finger 3).[47] **Greig cephalopolysyndactyly** syndrome is characterized by frontal bossing of the head, scaphocephaly, and hypertelorism in association with radial and ulnar polydactyly and variable syndactyly.

Fanconi Anemia is a recessive genetically heterogeneous condition with involvement of at least 15 different *FANC* genes.[48] Fanconi anemia patients with mutations in any of these genes share a characteristic clinical and cellular phenotype, and the 15 gene products appear to function in a common cellular pathway, termed the Fanconi anemia pathway.[48] The commonest manifestation of Fanconi anemia (75-90%) is mild to severe bone marrow failure in the first decade of life but patients are susceptible to many different neoplasms due to a high degree of genomic instability.[49] The diagnosis engages hand surgeons world-wide since half of children with Fanconi anemia have radial upper limb anomalies most often thumb hypoplasia. Along with café au lait spots and short stature, bilateral radial ray anomalies should always raise awareness for Fanconi anemia and initiate genetic testing.

TAR (OMIM#274000) Thrombocytopenia absent radius syndrome is the combination of a reduction in the number of platelets and absence of radius. The thumb is preserved which separates TAR from other syndromes with radial engagement of the upper limb. TAR has recently been shown to be caused by compound heterozygous mutations in the exon-junction complex subunit *RBM8A* (Rna-binding motif protein 8a) and two regulatory units of *RBM8A*.[50]

VACTERL (OMIM#192350) is an acronym used for the following seven congenital anomalies that often occur together: Vertebral anomalies, Anal atresia, Cardiac anomalies, TracheoEsophageal fistulas, Renal anomalies and Limb anomalies. A minimum of three anomalies in the spectrum is required for diagnosis. At present, no common etiology is known to cause VACTERL. A heterogeneous background is likely and include gene dose alterations, X-linked inheritance and mutations or deletions of the *ZIC3* gene (Zinc finger protein of cerebellum 3).[51,52] Maybe the lack of genetic cause is due to the phenotypically diversity of VACTERL or more complex genetic interactions than expected, e.g. TAR and compound heterozygosity.

2.5 CLASSIFICATION OF CONGENITAL UPPER LIMB ANOMALIES

Congenital upper limb anomalies (CULA) engage many medical specialities. Among pediatricians, geneticists and hand surgeons different classifications and approaches to understand the complexity of CULA have been used. Hand surgeons are naturally more interested in morphological classifications as they give better guide to treatment and geneticists are more interested in etiological classification as they can guide to genetic counselling. The first classification in 1949 by Birch-Jensen [53,54] categorized reduction deformities of the upper limb into radial or ulnar defects, split hands or amputations. Swanson developed the currently used classification of CULA in 1968.[55] It was internationally accepted by hand surgeons in the International Federation of Societies for Surgery of the Hand, IFSSH in 1983[55] and modified by Knight et al. in 2000.[56]

The IFSSH classification separates CULA into seven main categories. (Table 2) It aims to include all CULA. The seven main categories are subdivided according to the proximodistal level, the radial to ulnar level and the type of tissue (vascular, neurologic, connective, skeletal) involved by the CULA. Further subdivisions of the different categories vary between the different categories. The rule of the IFSSH classification is to classify according to the predominant anomaly if there is more than one anomaly in the limb.

Table 2

IFSSH Classification of Congenital Upper Limb Anomalies categories	
I	Failure of formation
II	Failure of differentiation of parts
III	Duplication
IV	Overgrowth
V	Undergrowth
VI	Constriction ring syndrome
VII	Generalized abnormalities and syndromes

The IFSSH classification does not include current knowledge on molecular causes of CULA. A new classification of CULA has been proposed; the OMT classification (Oberg, Manske, Tonkin). The OMT classification incorporates current knowledge on limb development and divides CULA into four main categories: malformations, deformations, dysplasias and syndromes. Tonkin et al. refined it in 2013 to make it internationally accepted. [57,58] The anomalies of **paper 1** have been reclassified by Ekblom et al. [59] in order to assess the OMT classification on a large population-based material.

Other authors have proposed a more structural and unclassifying approach to CULA. In the recording system by Luijesterburg et al. all features of CULA are described and recorded along the entire upper limbs without regards to other anomalies on the same limb.[60]

3 AIMS

The extensive molecular knowledge on genetic mechanisms of limb development is more detailed than the limited knowledge on genetic causes of human congenital upper limb anomalies, especially isolated anomalies. Continuous efforts are needed to identify underlying genetic aberrations in human congenital upper limb anomalies to meet the demands of parents, pediatricians, geneticists and surgeons working with CULA. The general aim of this thesis is to improve knowledge on genetic mechanisms and epidemiology in human congenital upper limb anomalies.
The specific aims are:

- In **paper I** to describe the incidence of congenital upper limb anomalies with subcategories in Stockholm County and to further explore occurrence among relatives, associated non-hand anomalies, gender and affected side for congenital upper limb anomalies with subcategories.

- In **paper II** to find and further explore the molecular cause of triphalangeal thumbs with radial polydactyly in a large family with autosomal dominant inheritance pattern.

- In **paper III** to refine genotype–phenotype correlations for patients with 17q22 deletions, investigate evidence for a clinically recognizable, distinct, 17q22 microdeletion syndrome and to identify contributing genes.

- In **paper IV** to find the molecular cause of X-linked recessive fusion of the fourth and the fifth metacarpals in three families, to test these mutations functionally and to study the possible association to heart disease

4 SUBJECTS AND METHODS

Table 3 and the following section provide an overview of the study participants and the methods used. Detailed information can be found in the corresponding papers.

Table 3

Paper	Scope	Research Questions	Data sources	Study Design
I	Epidemiology of Congenital Upper Limb Anomalies (CULA)	What is the incidence of CULA in the Stockholm County population?	Medical registries at hospitals in Stockholm, National Board of Health and Welfare, Statistics Sweden and Swedish Tax Agency for the period of: Jan 1997 - Dec 2007.	Retrospective total population study based on the IFSSH classification of CULA.
II	Mutations in the Zone of polarizing activity regulating sequence in radial polydactyly.	What is the genetic cause of radial polydactyly and triphalangeal thumbs in a family with dominant inheritance pattern?	Outpatient clinic, and Sanger sequencing in Stockholm. Transgenic mouse assay and transcription factor binding site prediction at UCSF.	Descriptive and functional study.
III	17q22 Microdeletion syndrome.	What are the common characteristics of individuals with microdeletions of 17q22 including *NOG*?	Outpatient clinics in Oxford, Columbia USA, Sao Paolo and Stockholm. Reverse phenotyping and array-CGH at KS.	Multicenter retrospective case report.
IV	*FGF16* mutations in X-linked recessive fusion of the fourth and fifth metacarpals.	What is the genetic cause of X-linked recessive metacarpal 4-5 fusions and is it related to heart problems in one family?	Outpatient clinics in Stockholm, Örebro and Malmö. Exome sequencing at Science for Life, Sanger sequencing and functional assay with Zebrafish morpholinos at KI.	Descriptive and functional study.

4.1 STUDY POPULATION AND RECRUITMENT

For **paper I** children born in Stockholm between 1st of January 1997 and 31st of December 2007 were included. We searched medical registers at all hospitals in Stockholm treating congenital upper limb anomalies for an ICD10 diagnosis coding between Q68.1 to Q87.4, corresponding to congenital upper limb anomalies. Three registers at the National Board of Health and Welfare (NBHW); the Swedish Medical Birth Register (SMBR), the Swedish Hospital Discharge Register (SHDR) and the Swedish Register on Congenital Malformations (SRCM) were also checked, through the SRCM. According to the Swedish Tax Agency there were 261 914 live births in the Stockholm county during the study period (51% males and 49% females). On the 31st of December 2007 the total population of Stockholm county consisted of 1 949 516 inhabitants according to Statistics Sweden and the Swedish Tax Agency. We excluded the diagnoses in table 4 due to late presentation, large span of clinical presentation and lack of upper extremity involvement, as well as extremely rare conditions. The exclusion did not influence incidence rates of each category or diagnosis (Appendix 1) but most likely had a minor impact on the overall incidence for CULA in the study. In total 562 children with 585 congenital upper limb anomalies were included in a database created for the purpose of **paper I** and for further studies.

Table 4

Excluded Diagnoses in Paper I	
1	Congenital radial head dislocation
2	Congenital tumorous conditions
3	Epidermolysis bullosa
4	Ichtyosis
5	Marfan syndrome
6	Madelung deformity
7	Arthrogryposis multiplex congenita

The proband of the family in **paper II** came to the department of hand surgery for bilateral reconstructive surgery of his duplicated and triphalangeal thumbs. There was an extensive family history of radial polydactyly and the pedigree showed autosomal dominant inheritance pattern. Blood and spare tissue samples for DNA preparation were obtained during surgery.

The first individual in **paper III** attended the outpatient clinic at the department of clinical genetics as part of a syndrome investigation. Array-CGH showed a 1.98Mb deletion on 17q22. To find more individuals, reverse phenotyping was used. In reverse phenotyping the genetic aberration determines which individuals to include. By searching databases and contacting departments of clinical genetics worldwide, we found six individuals with the same deleted region on chromosome 17q22. The phenotypes of these individuals were then compared for similarities and differences.
In **paper IV** the database created for **paper I** was used to find families with X-linked recessively inherited metacarpal 4-5 fusions. We included three families with a

confirmed X-linked recessive family history and metacarpal 4-5 fusions. Annerén et al. had previously published family 1 and the probands had been surgically treated at a department of hand surgery. The proband of family 2 was surgically treated at a department of hand surgery and blood sample for DNA preparation was obtained during surgery. No individual in family 3 was subject to surgical treatment.

4.2 CLASSIFICATION OF CONGENITAL UPPER LIMB ANOMALIES

For the purpose of **paper 1** three hand surgeons classified all 562 children with CULA according to the IFSSH classification. We carefully studied medical records for all children and available radiographs prior to surgery in 53% of the cases. All children were analysed with regard to gender, laterality, associated non-hand anomalies, occurrence among relatives, syndromes and previous surgery. Children with bilateral anomalies belonging to the same IFSSH category were recorded as one main anomaly. For 23 children, the right and the left side anomaly belonged to different categories and therefore these were counted as two anomalies. The material consisted of 585 anomalies in 562 individuals.

4.3 STATISTICS

Descriptive statistical analyses in **paper I** was performed with IBM SPSS Statistics 17.0 to calculate incidence (number of new individuals per live births per year) and not prevalence (number of existing individuals at a specific time) as end result. By dividing the number of anomalies with the number of live births per year and multiplying by 10,000 we calculated the incidence.

4.4 TISSUE HANDLING

For the purpose of this and future work we used a biobank at the department of clinical genetics, Karolinska University Hospital to store all DNA-samples. Blood and spare tissue samples were obtained during regular scheduled reconstructive hand surgery performed by hand surgeons from the department of hand surgery at Södersjukhuset. Samples were transferred from the department of hand surgery, Södersjukhuset or the operating theatre at Astrid Lindgren children´s hospital and DNA was prepared according to standard protocols. A total of 130 DNA samples from blood were obtained. For the purpose of investigating somatic mutations, mosaicism, we obtained tissue samples that otherwise would have been discarded during surgery from in total 70 patients.

4.5 ETHICS

Ethical approvals were obtained for all four studies of this thesis. For the genetic studies all individuals or their legal guardians signed informed consents. In **paper III** legal guardians of the studied individuals gave permission to publish facial photographs.

4.6 MUTATION SCREENING

4.6.1 Polymerase chain reaction, PCR

Polymerase chain reaction, PCR, creates multiple copies of a selected region of DNA. To specify what area to multiply, primers are designed on either side of the region of interest. Primers are oligonucleotides complementary to the DNA strand. To perform PCR the DNA template, primers, free nucleotides and DNA polymerase are placed in a PCR-machine. The PCR-machine raises and lowers the temperature in the sample and thereby the primers denature and anneal. The DNA-polymerase binds to the annealed primers and synthesise DNA-strands during the annealing period of every cycle. For every temperature cycle the region of interest is doubled until millions of copies are created.

4.6.2 DNA sequencing

With DNA sequencing the order of the nucleotides in DNA are determined. Frederick Sanger developed Sanger sequencing in 1975. In 1980 he was rewarded the Nobel prize in chemistry. Sanger sequencing involves production of DNA fragments of various lengths. Every fragment is terminated with an added dideoxynucleotide (ddATP, ddGTP, ddCTP and ddTTP). By lining up the fragments by length and read the last nucleotide one can read the DNA sequence of the original sample.

PCR and Sanger sequencing was used in **paper II** with primer sequences for the Zone of polarizing activity Regulating Sequence, ZRS from Lettice et al.[23]. In **paper IV** PCR and Sanger sequencing of *FGF16* was performed with seven primer pairs for the coding region of *FGF16* (exon 1, exon 2, exon 3) and the 1500bp up-streams region of exon 2 (intron 1) in order to confirm the nonsense mutation c.361G>T in *FGF16* detected by Whole Exome Sequencing and to perform segregation analysis in family 1. PCR and Sanger sequencing was also used to screen for mutations and perform segregation analysis in families 2 and 3. We used reference sequence for all *FGF16* exons and surrounding regions from NCBI refseq NW_003871101.3.

4.6.3 Array Comparative Genome Hybridisation, Array-CGH

Array-CGH is a genome wide technique used to screen for gene dose alterations. Array-CGH uses two genomes, a test and a reference, which is differently labelled with either red or green fluorescent dye and applied to a microarray. The two genomes compete to hybridise (attach) to the probes of the microarray. A scanner measures the fluorescent signal for every probe. The fluorescence ratio of the test and reference signals is determined at different positions along the genome, and gives information on the relative copy number of sequences in the test genome as compared to the used reference genome. The resolution of array-CGH depends on the size and amount of probes used for each location in the genome.

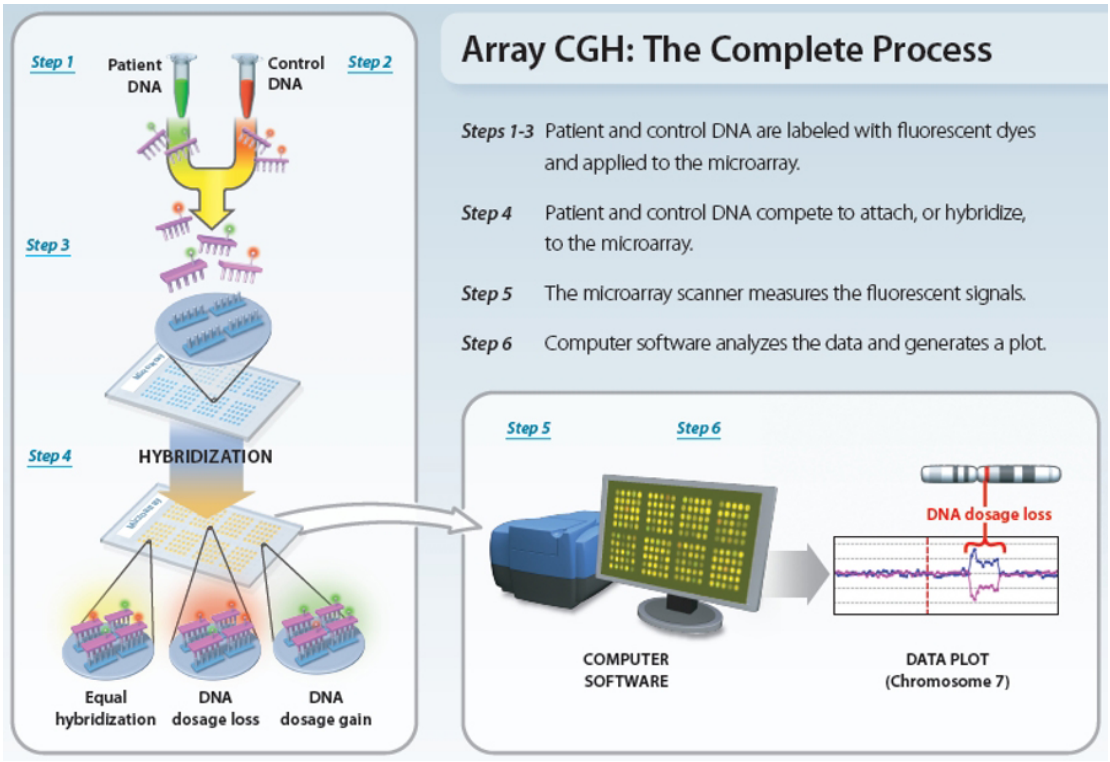

Figure 4. Adapted with permission from: Theisen, A. (2008) Microarray-based comparative genomic hybridization (aCGH). Nature Education 1(1):45

In **paper III** array-CGH was carried out using DNA isolated from peripheral blood for all six individuals. The effective mean resolution of the arrays was 16–54kb based on three to four consecutive oligonucleotides showing a change. Slide scanning was performed using an Agilent Microarray scanner. Data was extracted using Agilent Feature Extraction software (Agilent, Santa Clara, CA, USA). We used the OGT cytosure interpret software and the Agilent CGH Analytics software to analyze data.

4.6.4 Whole exome sequencing, WES

Massive parallel sequencing also known as second generation sequencing has revolutionised research in molecular genetics and now offers the possibility to screen individual genomes, exomes or customized panels of regions at nucleotide level. There are many high-throughput DNA sequencing techniques, but they all generate large numbers of short reads, fragments of DNA sequence. In WES all genes, the exome, of an individual are selected for sequencing. The noncoding parts of the genome are not. The laboratory part of WES with the Illumina system can be divided into three steps, Library preparation, amplifying and sequencing: The library of the DNA to be sequenced is prepared by fragmenting the genome into less than 600bp pieces. Adapters are ligated into each end of the fragments. Magnetic beads are then used to selectively capture all genes, the exome. Clustering: The adapter-ligated DNA is then washed over a surface coated with millions of oligos, the flow cell, that are complementary to the adapter-sequences. One end of the fragments ligate randomly to the complementary oligos on the surface. The fragments amplify thousand-fold into clusters on the flow cell through bridge amplification. Bridge amplification is specific to the Illumina system whereas other means of amplification are used in other systems. To sequence the fragments in the clusters a universal primer is annealed to the loose ends of the fragments at the site of the adapter sequences. Repetitive cycles follow

where fluorescently labeled nucleotides are added to the flow cells, imaged at and high-resolution and washed away. Base calling occurs gradually as the nucleotides are added to the templates. For every cluster one read is created.

WES was used in **paper IV** for the proband and his cousin in family 1 and the proband and his brother in family 3 on an Illumina HiSeq2000 as described above. Reads were mapped to the human reference genome (hg19) using Mosaik (v1.0.1388) (Michael Strömberg, unpublished, http://code.google.com/p/mosaik-aligner/, arXiv: 1309.1149 **[q-bio.GN]**). Duplicate read pairs were removed using Mosaik DupSnoop. Variants were called using the SAMtools package (v.0.1.18).[61] These were quality filtered (Q>=20), and annotated using ANNOVAR (version 2011 Oct 05)[62]. Variants were further filtered using ANNOVAR to remove those found at a 1000 genomes minor allele frequency (MAF) of 2% and above, as well as variants found in dispensable genes, truncated at a MAF of more than 1% in any 1000 genomes subpopulation, and variants not predicted damaging by PolyPhen2[63](P threshold of 0.7). Non-synonymous variants, indels and putative splice site variants were retained. Remaining variants present in related affected individuals, but not found in a local cohort of 265 individuals with unconnected indication, were shortlisted.

4.7 FUNCTIONAL STUDIES

No human genome is exactly the same. As the possibilities to discover variations in genomes increase day by day, it becomes increasingly important to differ between normal and pathologic variation in human genomes. Functional assays are one way of testing whether a discovered variant is disease causing or not. In this thesis we have, in collaboration with other researchers, used transgenic mice and computational transcription factor binding site prediction in **paper II** and Zebrafish morpholinos in **paper IV**.

4.7.1 Transgenic mice

The functional impact of the 13bp ZRS insertion in **paper II** was tested with a Lac-Z enhancer assay in mice. ZRS with the 13bp insertion was PCR amplified from individual II/1 with primers carrying *XhoI* and *ApaI* restriction sites and cloned into the *Hsp68-LacZ* enhancer assay vector[64] This vector has an Hsp68 minimal promoter that is not sufficient to drive reporter expression without the presence of a functional enhancer in front of the LacZ reporter gene. The wild type enhancer assay vector was generated by removing the insertion from the ZRS603ins13 *Hsp68-LacZ vector*. Mice embryos were injected with the vector and at embryonic (E) day 11.5, stained for Beta galactosidase.

4.7.2 Transcription factor binding site (TFBS) prediction

To understand how the 13bp insertion in ZRS reported in **paper II** could affect the expression of SHH in the developing limb the mutant ZRS sequence was screened for TFBS differences from the wild-type human ZRS sequence in a computational analysis. 345 TFBS motifs from the UniPROBE database[65] as well as TFBS compiled

from the literature including *TBX5* (OMIM#601602) *TBX6* (OMIM#602427), the *PBX-MEIS1* complex (OMIM#602100, 601739) and the *MEIS1-HOXA9* (OMIM#142956) complex was used. Binding sites were predicted by scanning DNA sequences for motifs using an implementation of the MATCH algorithm[66]

4.7.3 Zebrafish morpholino

In **paper IV** the functional impact of *FGF16* loss was evaluated with Zebrafish (Tupfel longfin, TL) morpholinos, MO. Two non-overlapping MOs blocking either translation or splicing of *FGF16* were designed against the start AUG or the exon 2/Intron 2 splice junction respectively.

To ascertain mis-splicing of the pre-mRNA, RNA was extracted from uninjected TL or FGF16 MO injected TL embryos and cDNA was synthesized. The resulting cDNA primers spanning the splice junction were designed and used to amplify a 363 bp PCR product from 3dpf zebrafish embryos. The resulting product was Sanger sequenced and the introduction of a frameshift nonsense mutation was confirmed.

For mRNA rescue experiments, full length FGF16 mRNA was synthesized and cloned into the pCS2+ vector. The plasmid was linearized at the 3' end via enzymatic digestion with Not1 and used as a template for the generation of capped poly-adenylated mRNA.

For rescue experiments MOs and RNA were injected into the yolk of 1-2 cell embryos. After injection, embryos were screened for a reduction of pectoral fins and abnormal gross morphology at 3-4 days post fertilization. All injections were repeated at least twice and similar results were pooled after testing for homogenity of experimental groups using $\chi 2$ test of homogeneity. The significance in difference of phenotypic penetrance was analysed using the $\chi 2$ test.

5 RESULTS

Table 5 and the following sections provide an overview of the epidemiological and genetic results in this thesis. Detailed information can be found in the respective papers.

Table 5

Paper	Research Question	Study Design	Results	Conclusion
I	What is the incidence of CULA in the Stockholm County?	Retrospective total population study based on the IFSSH classification of CULA.	In Stockholm County between 1997 and 2007 the incidence rate of CULA was 21.5 per 10,000 live births. Incidence rates and further data on CULA is subdivided and reported.	In Stockholm County, the incidence of CULA is 21,5/10000 live births, which is comparable to the only other total population study so far.
II	What is the genetic cause of radial polydactyly and triphalangeal thumbs in a family with dominant inheritance pattern?	Descriptive and functional study.	The 13 base pair insertion, ZRS603ins13, causes radial polydactyly in a family with dominant inheritance pattern.	Insertions can add transcription factor binding sites in ZRS and thereby cause ectopic anterior expression of SHH in the limb bud and radial polydactyly
III	What are the common characteristics of individuals with microdeletions of 17q22 including NOG?	Multicenter retrospective case report.	Six individuals with microdeletions of 17q22 including NOG have similar facial characteristics, multiple bone and joint problems including symphalangism and intellectual disability.	The similarities between the 6 individuals represent a novel 17q22 microdeletion syndrome.
IV	What is the genetic cause of X-linked recessively inherited metacarpal 4-5 fusions and is it related to heart problems in one family?	Descriptive and functional study.	In three families, a nonsense, a splice site and a non-synonymous mutation in *FGF16* segregated with X-linked recessive metacarpal 4-5 fusions and in the family with nonsense mutation; heart disease. Knockdown of zebrafish Fgf16 resulted in half size fin buds and heart oedema.	Mutations in *FGF16* cause x-linked recessive metacarpal 4-5 fusions and truncating nonsense mutations in *FGF16* could contribute to heart disease

5.1 EPIDEMIOLOGICAL RESULTS

5.1.1 Incidence relative frequency in Stockholm

The total incidence of children with CULA in Stockholm was 21.5 children per 10,000 live births during the 11-year period studied.

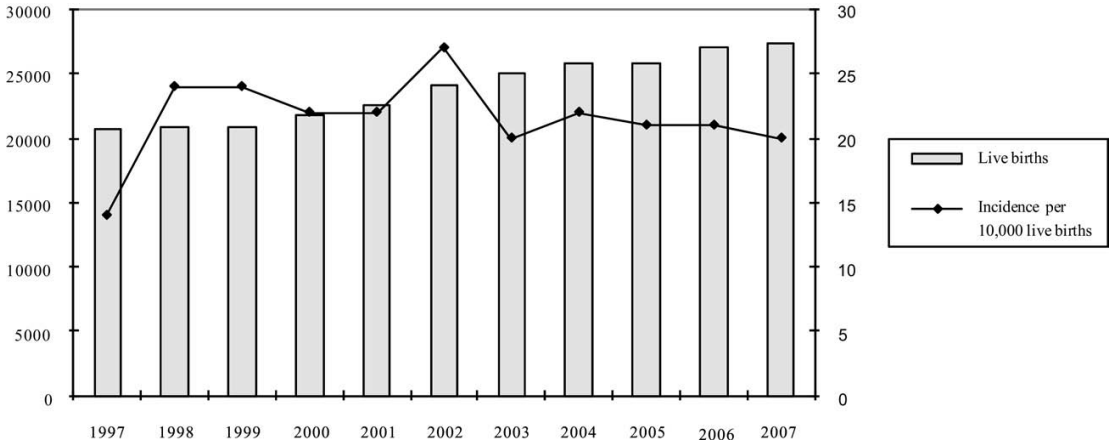

Figure 5. Incidence of children with CULA per 10,000 live births per year and number of live births per year from 1997 to 2007 in the Stockholm region. From Ekblom et al. [67] with permission from publisher

In total, 562 children with 585 CULA were identified in this study. Twenty-three of the children had bilateral CULA belonging to different IFSSH categories.

The failure of differentiation category was the largest (276 children) of the seven main categories of the modified version of the IFSSH classification, followed by duplications (155 children) and failure of formation (103 children). The categories; overgrowth (10 children), undergrowth (18 children), constriction ring syndrome, and generalized skeletal abnormalities and syndromes were much less frequent.

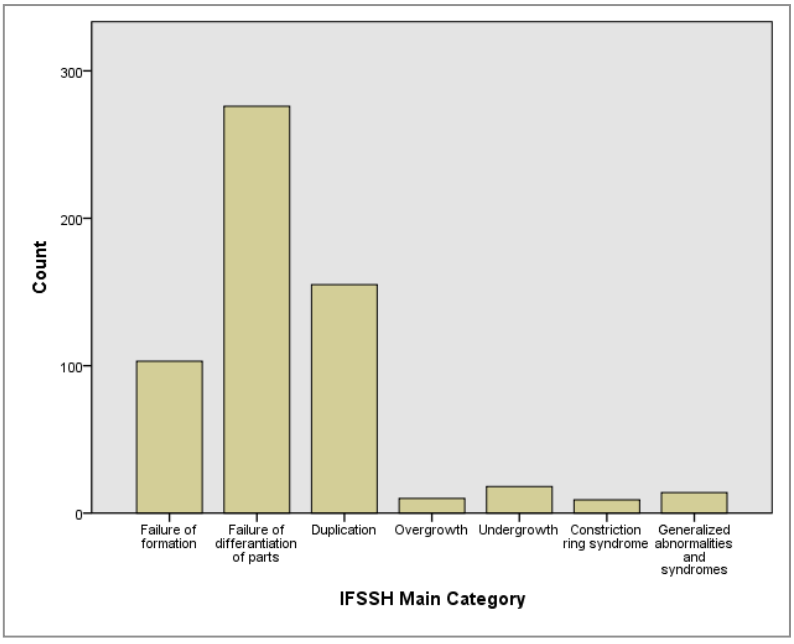

Figure 6. Distribution of children in the seven IFSSH main categories in paper 1, from Ekblom et al.[67] with permission from publisher

5.1.2 Gender

There were more boys (54%) than girls (46%) with CULA in this study compared to live births in the Stockholm county (51% boys vs 49% girls). The male predominance was most pronounced in the categories of failure of formation and duplication. On the other hand, there was a predominance of girls in the categories of undergrowth and constriction ring syndrome, although the number of children in these two categories was limited.

5.1.3 Laterality

In total there was a slight predominance of the left side only (29%) compared with the right side only (32%). The anomalies affected both sides in 230 of the 585 anomalies (39%). 207 children of 562 had a bilateral anomaly (37%).

5.1.4 Occurrence among relatives

In 99 of 562 children (18%) there was a known occurrence of limb anomalies among relatives. Table 6 shows CULA with the most frequent occurrence among relatives.

Table 6

Distribution of CULA with above average (18%) frequency of occurrence among relatives.

Anomaly	*Number of children with anomaly*	*Number of children with occurrence among relatives*	*Percent of children with occurrence among relatives*
Symphalangism	1	1	100
Central polydactyly	4	3	75
Cutaneous syndactyly	36	19	53
Wind-blown hand	5	2	40
Complex syndactyly	13	5	39
Cleft hand	8	3	38
Proximal radio-ulnar synostosis	3	1	33
Triphalangeal thumbs	10	3	30
Brachydachtyly	11	3	27
Clinodactyly	31	8	26
Deviated finger without bony deformity	4	1	25
Ulnar polydactyly	92	22	24
Radial polydactyly	59	14	24
Camptodactyly	40	8	20

5.1.5 Associated non-hand anomalies

130 of the 562 children (23%) had associated non-hand anomalies. Table 7 presents the diagnosis in which associated anomalies were most frequent. Table 8 presents the distribution of the associated non-hand anomalies in order of frequency. Anomalies in the lower limbs were most common.

Table 7

Distribution of CULA with above average (23%) frequency of associated anomalies.			
Anomaly	*Number of children with anomaly*	*Number of children with associated anomaly*	*Percent of children with associated anomaly*
Radius hypoplasia/aplasia	13	11	84,6
Sprengel deformity	5	4	80
Failure of differentiation, skeletal involvement, shoulder level	2	1	50
Brachysyndacyly	2	1	50
Thumb hypoplasia	20	9	45
Cutaneous syndactyly	36	16	44
Constriction ring syndrome	9	4	44
Complex syndactyly	13	5	39
Macrodactyly	3	1	33
Brachymetacarpia	3	1	33
Ulnar polydactyly	92	27	29
Hemihypertrophy	7	2	29
Transverse arrest	22	6	27
Poland syndrome	4	1	25
Cleft hand	8	2	25

Table 8, from Ekblom et al. [67] with permission from publisher. * Rounding.

Distribution of Associated Non-hand Anomalies in 562 Children in order of frequency			
Associated anomaly	*Number of children*	*Percent of 562 children*	*Percent of 252 Associated non-hand anomalies*
Lower limb	54	9.6	21.5
Syndromes	38	7.1	14.7
Circulatory system	30	5.3	11.9
Head and neck	26	4.6	10.4
Urogenital	25	4.4	10.0
Digestive system	25	4.4	10.0
Central nervous system	19	3.4	7.6
Vertebral column	12	2.1	4.8
Respiratory system	10	1.8	4.0
Body wall	9	1.6	3.6
Skin	4	0.7	1.6
Total number of children with associated non-hand anomalies	130	23.1	100.1*

5.1.6 Selected diagnosis-specific observations

Congenital trigger digit or thumb was the most frequent diagnosis in the whole study (115 children). Incidences of "Top ten" diagnoses are shown in figure 7 below. Appendix 1 lists the incidence, relative frequency, gender distribution, laterality of the affected side, occurrence among relatives, and associated non-hand anomalies for all anomalies in the study

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5.1.6.1 Transverse arrest

Of the 22 transverse arrests reported none were bilateral and no child had any relative with an upper limb anomaly. 6 of the 22 children had an associated anomaly. The most common level of arrest was in the forearm (10) and the wrist (5).

5.1.6.2 Radial ray deficiency

Of 33 radial ray deficiencies 20 were isolated thumb deficiencies and 13 engaged radius with either hypoplasia or aplasia. Gender distribution and the incidence of associated non-hand anomalies differed between isolated thumb deficiencies and radial hypoplasia/aplasia. For isolated thumb deficiencies, there was a female preponderance (12 of 20), but for the radial hypoplasia/aplasia children there was a male preponderance (9 of 13). Associated non-hand anomalies were less common in the isolated thumb deficiencies (9 of 20) than in radial hypoplasia/aplasia (11 of 13).

There was a known occurrence of limb anomalies among relatives in 2 of 20 children with isolated thumb deficiencies, but no limb anomalies among relatives to children with radial hypoplasia/aplasia.

5.1.6.3 Ulnar ray deficiency

In 25 children with ulnar ray deficiency 17 were isolated to the ulnar hand and in 8 children ulna was involved with either hypoplasia or aplasia. In the latter group, 2 children had a severe deformity with a short humerus. These two children were classified as ulnar ray deficiency type V according to Goldfarb et al., and not as intersegmental arrest (phocomelia).[68] For ulnar ray deficiencies there were a preponderance of boys (21 of 25).

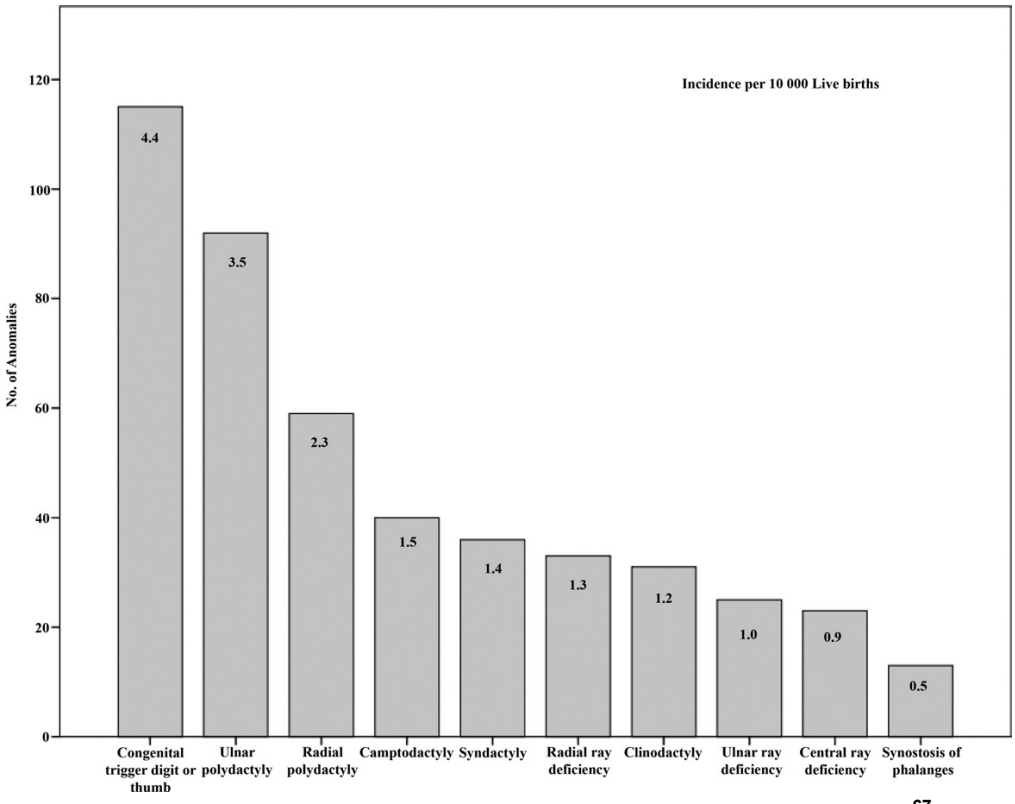


Figure 7. Incidence of "Top ten" Diagnosis in paper I. From Ekblom et al. [67] with permission from publisher

5.1.6.4 Central ray deficiency

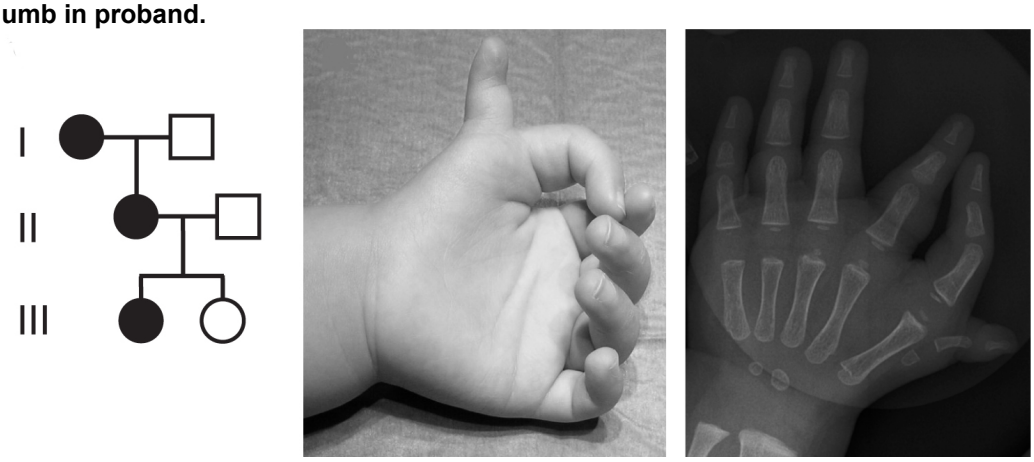
Eight of 23 children with central ray deficiency were classified as having cleft hand and 15 as symbrachydactyly. There was a male predominance of both cleft hand and symbrachydactyly. For the children with symbrachydactyly, most were on the left side only and one individual was bilateral. Occurrence among relatives was more common among children with cleft hands (38%) compared with those who had symbrachydactyly (7%). One child with symbrachydactyly had an ipsilateral hypoplasia of the pectoral muscles (i.e., Poland syndrome). Otherwise there were no associated anomalies in the symbrachydactyly group.

5.2 GENETIC RESULTS

5.2.1 ZRS Insertion

In **paper II** clinical examination of the proband showed bilateral non opposable symmetrical triphalangeal thumbs with an extra hypoplastic radial thumb containing two phalanges and one metacarpal—PPD type II (MIM#174500), figure 8. Neither the proband nor any other family member had any involvement of the lower limbs. In six-generations of the probands family there were 78 individuals, 18 of whom were reported to be affected. DNA samples were available from three affected and three unaffected individuals in three generations and clinical examinations were performed on the probands parents and sister (figure 8).

Figure 8. Left: Pedigree showing the six family members which DNA-samples were available from. Middle and right: Triphalangeal thumb with a hypoplastic radial extra-thumb in proband.



Mutation-screening of the 2.1 kb region that encompasses the ZRS and a nearby region where mutations have been shown to cause polydactyly in dogs (pZRS)[69] showed that all affected individuals carried a heterozygous 13 bp insertion (TAAGGAAGTGATT) starting at position 603 of the ZRS sequence[23], position g.106934 within reference sequence NG_009240.1. None of the three tested unaffected members carried the insertion that was named: ZRS603ins13.

Transcription factor binding site, TFBS, prediction showed that the insertion creates two major sites that match the binding preferences for several transcription factors, including some that are important in limb development. Of the detected TFBS: Engrailed1 (EN1), TBX5, TBX6, SOX8, and multiple HOX genes including HOXA9, HOXB8, and HOXC are known to be involved in limb development. No limb-related TFBS was predicted to be disrupted by the insertion.

Transgenic mice carrying an HSP68-LacZ enhancer assay vector with the wild-type ZRS sequence recapitulates the normal SHH expression pattern in E11.5 mouse embryos with expression of the reporter gene in the posterior region of the limb bud in seven out of eight independent transgenic mice. With the insertion, four out of six LacZ-positive embryos had limb expression and all four of these embryos showed ectopic anterior and posterior expression instead of the normal posterior-restricted

ZPA expression pattern, figure 9. The expression was limited to the mesoderm of the limb bud and absent from the apical ectodermal ridge with staining at the anterior and posterior sides. Although the four embryos did not show the same intensity of staining each embryo had anterior expression that was nearly as strong as the posterior expression. The reproducible anterior expression in ZRS603ins13 embryos was consistent with the enhancer expression pattern observed in similar assays with ZRS mutations that have been shown to cause polydactyly.[23,70,71] We observed no other consistent expression patterns in these embryos.

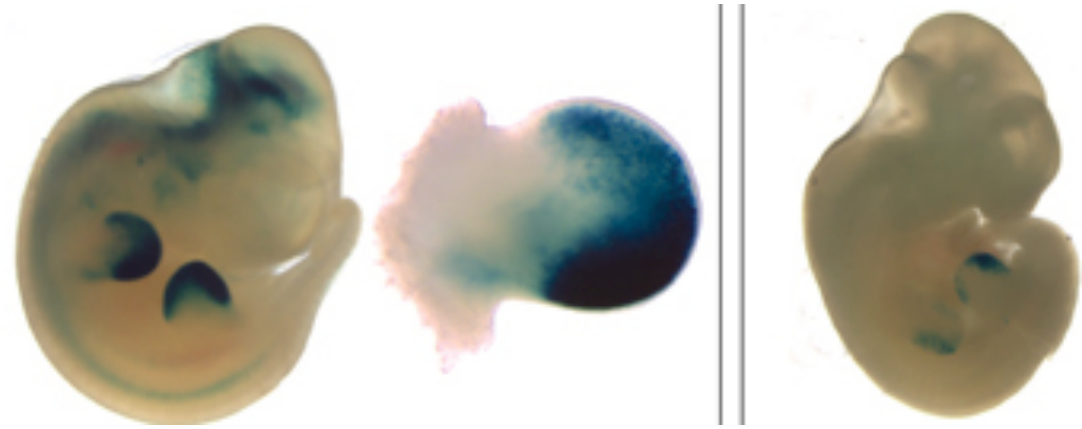


Figure 9, Whole-mount and dissected forelimb show anterior limb LacZ expression driven by ZRS603_604ins13 in transgenic E11.5 mouse (left) compared to predominantly posterior expression driven by wtZRS in the forelimb (right).

5.2.2 17q22 Microdeletions

The shared clinical features for each individual in **paper III** represent evidence for a clinically recognizable 17q22 microdeletion syndrome. Photos of faces and hands are shown in figures 10 and 11 (except for individual six). The syndrome includes microcephaly, intellectual disability, visual impairment, distinctive facial features such as thin border of upper lip, upslanting palpebral fissures, micrognathia, hypertelorism and *NOG*-related bone and joint-features such as symphalangism, conductive hearing loss and joint-contractures. Five patients displayed urogenital malformations. Patient 3 and 6 had cryptorchidism and penile chordee. Patient 2 had hypogonadotrophic hypogonadism, absent uterus and a rudimentary vagina. Patients 4 and 5 had duplicated renal collective systems. In Array-CGH revealed microdeletions involving the *NOG* gene on 17q22 in all individuals. In patients 1, 2, 3 and 6 they were de novo. The deletions involved an 8 184 525-bp region (chr17:48389130-56925730, hg19), with a maximal deletion size of 8 184 525 bp and a minimal size of 1 860 732 bp. The commonly deleted region of 237 304 bp (chr17:54875445-54638141,hg19) encompassed the *NOG* gene and an open reading frame gene called *C17ORF67*. Neither deletions nor duplications of this region have been reported in the region in control individuals.

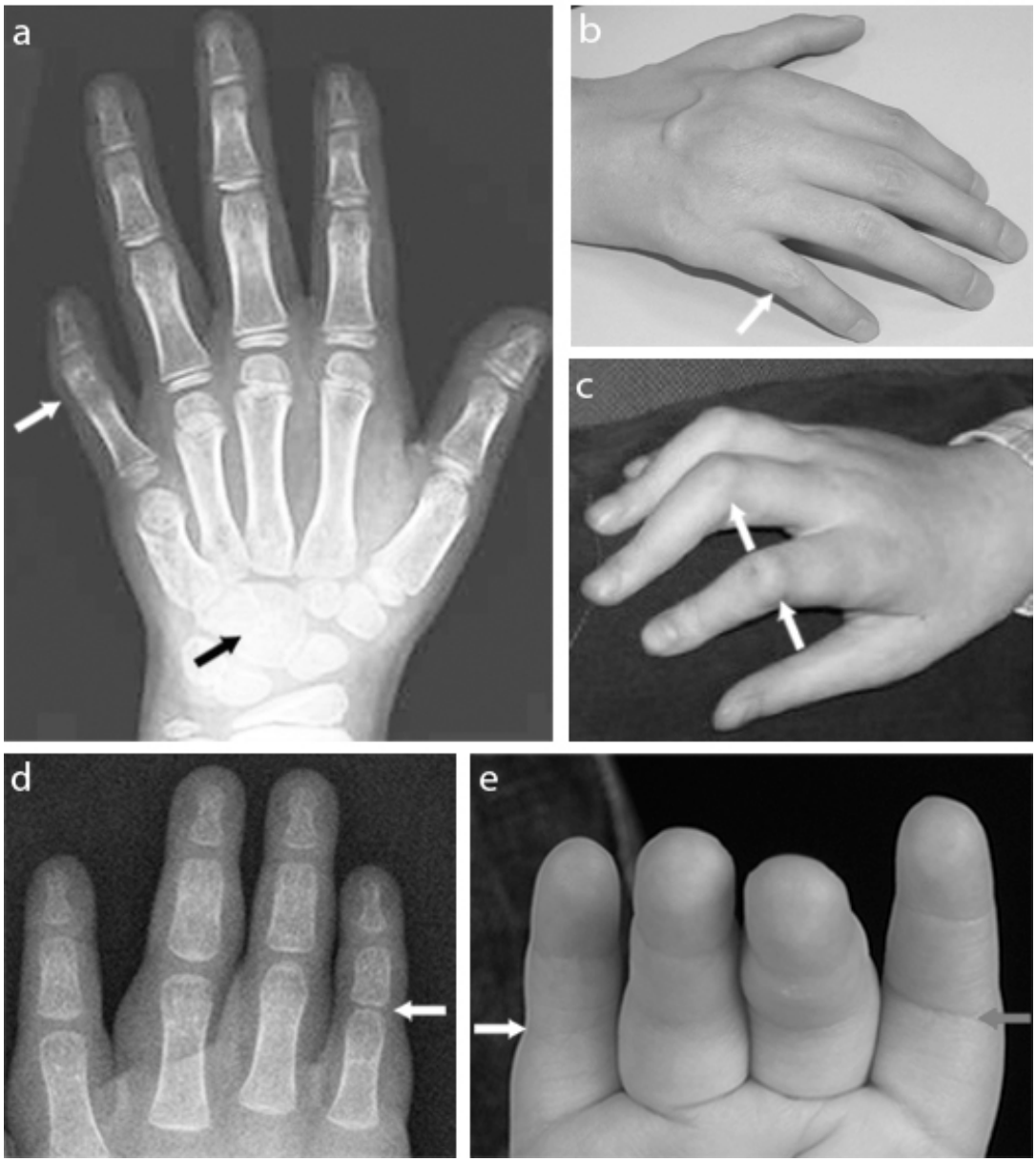


Figure 10 Symphalangism in the 17q22 microdeletion patients showing as: a **Ankylotic proximal interphalangeal joint in the fifth digit in patient 4 (white arrow). Note also the fusion of the capitate and hamate bones (black arrow).** b **and** c **Stiff proximal interphalangeal joints with reduced dorsal creasing in patient 3(b) and patient 2(c).** d **Reduced joint space in the fifth digit proximal interphalangeal joint in patient 1.** e **Stiff fifth finger proximal interphalangeal joint with absence of volar crease (white arrow) compared to normal crease in the second digit proximal interphalangeal joint (grey arrow).**

Describing hands in NOG-related symphalangism spectrum disorders[42] requires attention to details especially in symphalangism, where the clinical appearance can differ from radiographic findings. Patient 1 had stiff proximal interphalangeal joints and absent flexion creases at clinical examination, but radiographs at one and a half years of age were almost normal except for narrowing of the joint space of the proximal interphalangeal joint of the fifth digit, figure 10. There could be many reasons for these findings. Firstly, a radiograph of the hands at early age cannot adequately delineate the joint because of lacking calcification of the skeleton. Secondly, the gap showing at the proximal interphalangeal joints of the radiograph

may be filled with uncalcified cartilage and the ankylosis may be visible only when calcification is complete.[72] Moreover, the proximally placed thumbs reported previously require attention. Radiographs for patients 1, 2 and 4, figure 10a, revealed a short first metacarpal but the base of the first metacarpal and the carpometacarpal-joint were not proximally placed as one might have suspected at clinical examination and as described in previous reports.

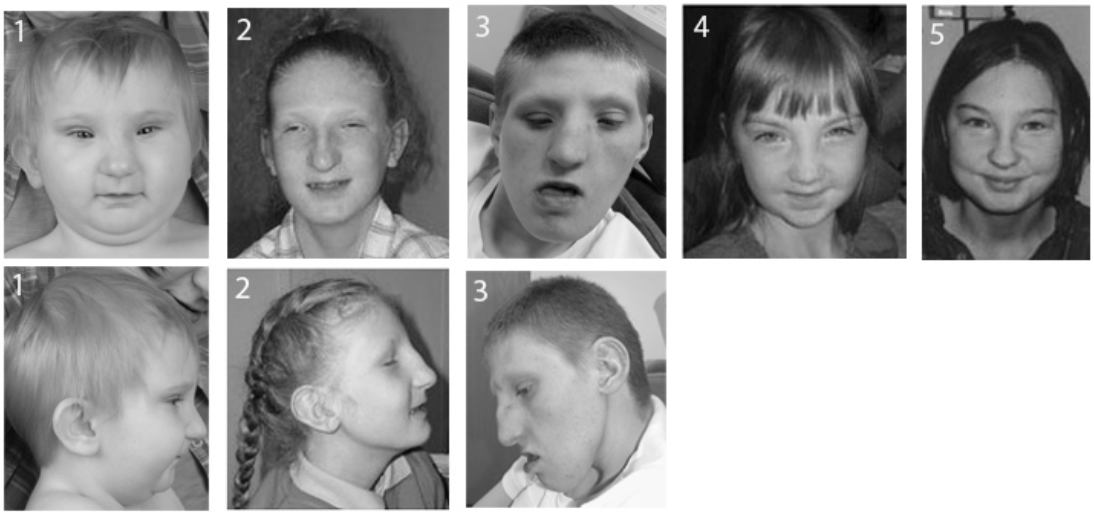


Figure 11 Facial characteristics of the 17q22 microdeletion patients including: Maxillary hypoplasia 2-5, short philtrum 1-5, thin border of upper lip 1-5, micrognathia 1,4,5, large bulbous nose 1-5, hypoplastic alae nasi 2-5, prominent columella 1-5, large dysplastic ears 1, upslanting palpebral fissures 1,3-5, narrow palpebral fissures 1-5, epicanthal folds 1-2,4-5, strabismus 1-2, hypertelorism 1,3-5 and blepharophimosis 1-3.

5.2.3 *FGF16* mutations

In the three families of **paper IV,** the pedigree showed an X-linked recessive inheritance pattern. Clinical examination of the probands showed ulnar deviation and hypoplasia of the fifth digits to various extents. Radiographs showed fusion between the fourth and fifth metacarpals (figure 12) MF4 (OMIM#309630) to various extents. Some of the fifth digits were fixed in 45 degrees of ulnar deviation at the level of the metacarpophalangeal joint without active or passive adduction. Flexion and extension in the digits was normal. In family 1 three male cousins (IV-6, IV-10, IV-12) who were later found to have a nonsense mutation in *FGF16* showed variable severity of MF4, figure 12. The least severely affected, IV-6, had a broad proximal fifth metacarpal bone and a minor overall shortening of the fifth digit. None of the family members in the three families reported any other malformations. However, eight of the *FGF16* mutation-carrying members of family 1 reported cardiac complaints. All three families harbored segregating *FGF16* mutations revealed by whole exome sequencing and Sanger sequencing, the most severe being a c.361G>T (p.G121*) nonsense mutation in exon 2 of *FGF16* in family 1.

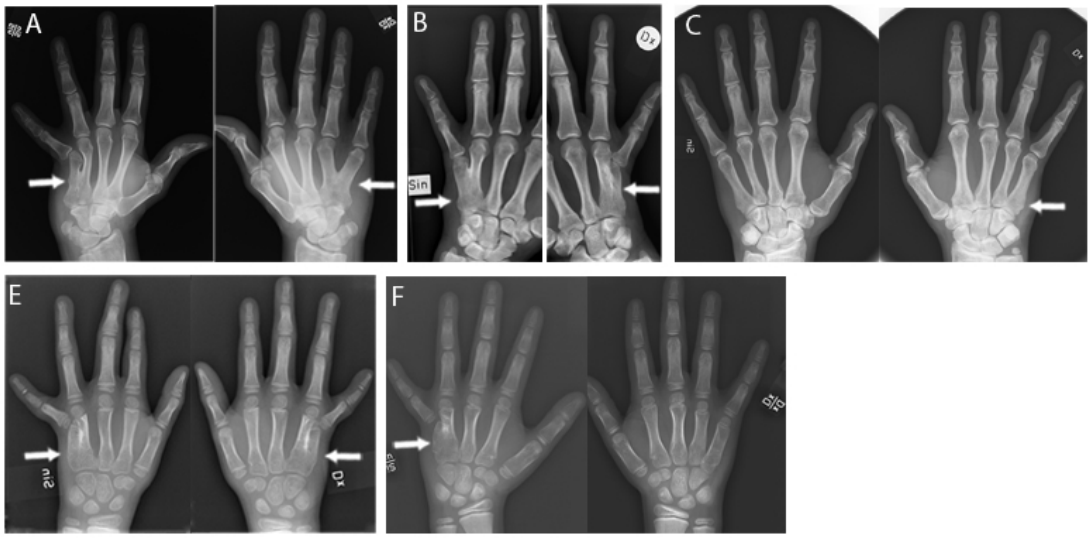


Figure 12. Phenotype variability of MF4 in three cousins in family 1 with the nonsense mutation (A-C). White arrows indicate fusion of the fourth and the fifth metacarpal in (A) and (B) and a broader proximal part of the fifth metacarpal in (C). White arrows also indicate fusion of the proximal part of the fourth and the fifth metacarpals in the probands of family 2 (E) and family 3 (F).

FGF16 is highly conserved among different species including the position for the three mutations. For the c.203G>T (p.R68L) transversion detected in family 3, *in silico* prediction with Panther Classification System[73] indicated that the variant is possibly damaging. Evaluation of abnormal splicing of *FGF16* with a splice blocking morpholino (sbMO) resulted in a severe reduction in the size of the pectoral fins, designated here as a class I phenotype, in 46% of 101 embryos injected (figure 13) similar to a previous report[74]. Thirty percent of the 101 embryos injected showed a more severe gross malformation of the body in addition to the fin phenotype designated as class II phenotype (figure 13). All affected embryos developed heart oedema (76% of the 101). Also, injection of MO against the translational start site to block ribosome binding gave similar results including the presence of heart oedema. The phenotype was rescued when *fgf16* MO were co-injected with full length human *FGF16* mRNA. Taken together these results confirm the role of *fgf16* in both fin and heart development of the zebrafish.

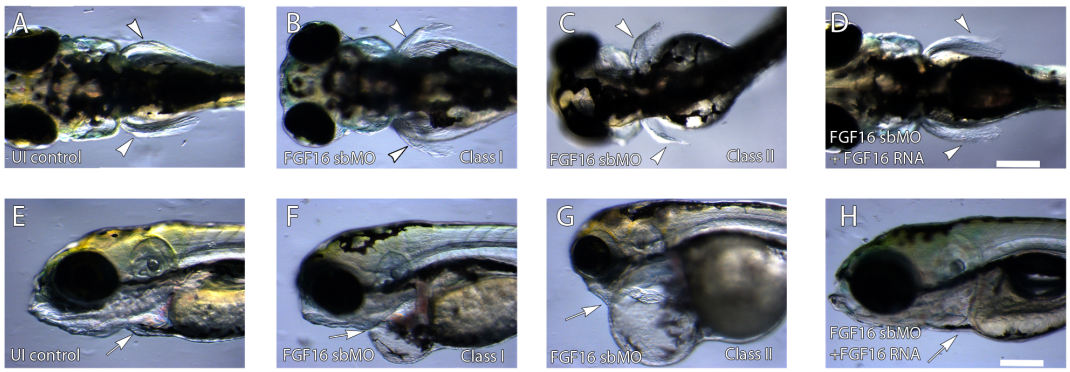


Figure 13: Knockdown of FGF16 results in reduced fins and heart malformations. Zebrafish embryos at 4 days post fertilization (dpf). Dorsal views of the head are shown in A-C and lateral views in E-F. Rostral is to the left in A-G. Injection of *fgf16* sbMO results in a severe reduction in fins (filled arrowheads in B, C) when compared to uninjected control embryos (A) or embryos co-injected with FGF16 sbMO and human *fgf16* wild type RNA (D). *Fgf16* MO injected embryos also show a malformation of the heart (filled arrow in F, G) compared to both uninjected (E) and embryos co-injected with sbMO and RNA (H).

6 DISCUSSION

6.1 EPIDEMIOLOGY PAPER I

In **paper I** the overall incidence of congenital upper limb anomalies, CULA, in Stockholm County between 1997 and 2007 was shown to be 21,5 per 10000 live births. This figure is similar as that obtained in the only other population-based study published so far (Giele *et al*: 19.7/10000 live births).[75] The lower incidence figures found in older studies, 16/10000[76] and 18/10000[77], are probably due to the fact that these studies were hospital-based and therefore many minor anomalies may have been missed. Our careful search for cases might have generated a somewhat higher incidence of CULA. Pre-requisites for performing an epidemiological total population study are good in Sweden with national registration of all inhabitants by the Swedish tax agency and the registration of anomalies by the National Board of Health and Welfare (NBHW). Thereby more accurate incidence figures could be provided compared to studies based on data from isolated specialized centres that rely on their treated patients only. Nevertheless, we encountered problems when comparing data from all Stockholm hospitals treating CULA and the three registers at NBHW (figure 14). Many of the cases identified in the hospital-based registers were not found in the registers held by NBHW. Also, we could not confirm many of the CULA cases identified in NBHW registers. The discrepancy could be due to the fact that some conditions are not detectable at birth, others are difficult to separate from traumatic conditions and some might not have been diagnosed properly. Since we included only cases verified in medical records at the Stockholm hospitals, the incidence of some conditions, especially ulnar polydactyly and syndactyly, may have been underestimated.

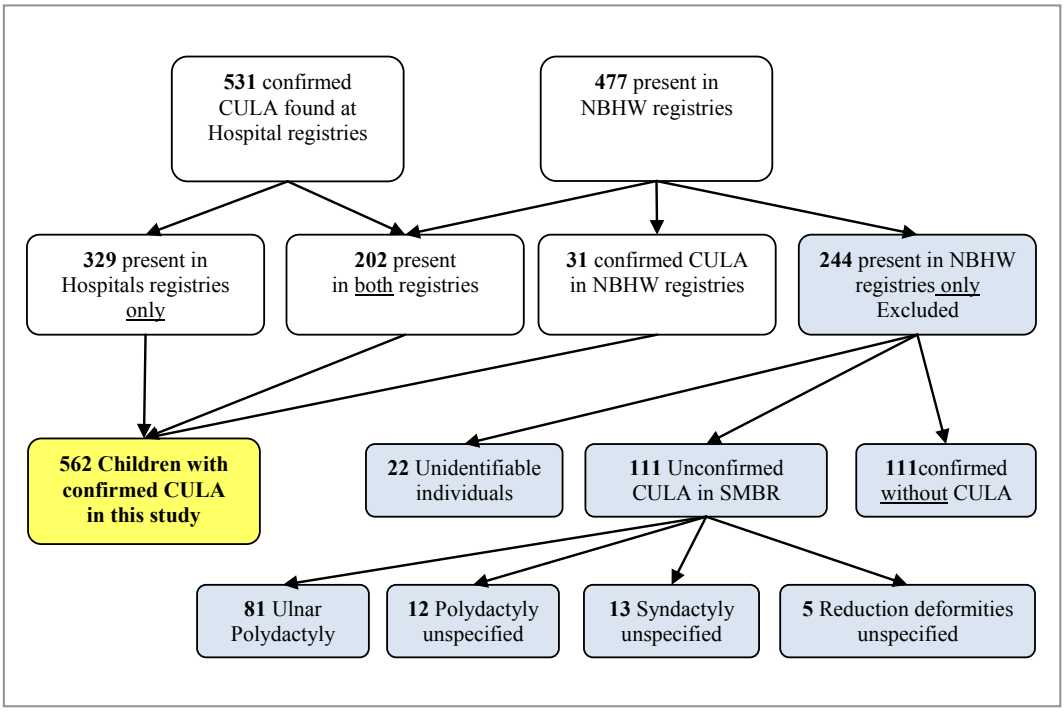


Figure 14 Disparity between registries. Ekblom et al. [67] with permission from publisher.

The use of any classification requires broad knowledge on details in phenotype presentation. In the hand many features often occur simultaneously, e.g. syndactyly, polydactyly and clinodactyly. The IFSSH classification allows only the most significant feature of the CULA to be classified, which is one of its many drawbacks. Another obstacle is that it only describes morphology and makes no use of current genetic and embryological knowledge on limb development. The recently developed OMT (Oberg Manske Tonkin) classification makes use of updated knowledge on limb development and includes the three embryological axes of limb bud development.[57] The individuals in **Paper I** were reclassified by our group to evaluate and assess the OMT classification.[59]

There are other approaches to CULA than the IFSSH and OMT classifications. These mainly aim to describe CULA from a morphological and surgical perspective, regardless of etiological considerations. Jones et al. describe the lack and presence of digits along the radial and ulnar axis with the purpose of evaluating the need for pediatric hand surgery, e.g. toe to hand transplantation.[78] Luijsterburg et al. has developed a non-classifying recording method in which every feature of the CULA is recorded separately.[79]

For some diagnoses the IFSSH classification has built-in controversies: Clinically, there are small differences between cutaneous syndactyly and complex syndactyly with synostosis of the distal phalanges. In the IFSSH classification these conditions are classified into two different subcategories: *Failure of differentiation*, *soft tissue involvement* and *Failure of differentiation, skeletal involvement*. The distinction between symbrachydactyly, brachysyndactyly and transverse arrest is also widely debated among hand surgeons.[80-83] In **paper I** we used the presence of nails as a criterion for symbrachydachtyly. Another difficult IFSSH category is *Undergrowth*. It groups various types of underdevelopment with different etiologies. Giele et al. classified thumb hypoplasia as *Undergrowth.*[75] In **paper I**, thumb hypoplasia was classified as *Failure of formation, longitudinal arrest, radial ray,* which may be more developmentally correct.[84] For Poland anomaly with absence or hypoplasia of the pectoral muscles and an ipsilateral hand anomaly, problems also arise with the IFSSH classification since it is not specified whether the hand or the shoulder anomaly should be considered the main anomaly. We classified according to the hand anomaly in **paper I**. These classification controversies make comparisons between different studies difficult.

A good reason for retrospective studies of large patient materials is to evaluate whether a clinical opinion is correct or not. For example, it was previously the clinical belief, at our unit, that transverse arrest seldom was associated with other anomalies. However, our population-based study could show that as much as 27% of individuals with transverse arrest also had an associated anomaly. The general table (appendix 1) provides good counselling information about gender, laterality, associated anomalies and occurrence among relatives for different types of CULA.

6.2 GENETIC PAPERS II-IV

Since the highly conserved region ZRS[85] (also called MFCS1[25]) in intron 5 of the *LMBR1* gene was shown to have enhancer activity, regulate SHH expression and cause preaxial polydactyly in mice limbs, several point mutations in ZRS have been shown to cause human limb anomalies (reviewed by VanderMeer et al.[26]). The point mutations in the ZRS cause a range of preaxial polydactyly phenotypes and are scattered among the approximately 1kb long region of ZRS. Duplications in the ZRS also have been shown to cause preaxial polydactyly phenotypes but of more complex character such as Triphalangeal thumb polysyndactyly syndrome. There is no clear relationship between the location of the mutations in ZRS and the severity of the phenotype.

The ZRS603ins13 mutation described in **paper II** is the first insertion in ZRS and it provides clues about the mechanism on how mutations in ZRS cause anterior expression in the limb bud. Previously it has been speculated that ZRS mutations may disrupt transcription factor binding sites (TFBS) but in two studies increased affinity for proteins have been seen for two mutated ZRS sequences.[86,87] The ZRS603ins13 mutation in **paper II** results in the creation of two motifs on the DNA strand that were predicted to bind multiple transcription factors known to be expressed in early limb development including TBX5, TBX6, EN1, SOX8, and multiple HOX genes. Of these, EN1 (OMIM#131290) is of particular interest since it is a homeodomain-containing transcription factor involved in ventral development of the limb. After the publication of **paper II** the sequence AGGAA$^{G}/_{A}$T occurring five times in the wild type ZRS has been shown to bind ETS (E26 Transforming Specific) transcription factors in the limb bud.[27] Two reported point mutations and the ZRS603ins13 of **paper II** adds one extra AGGAA$^{G}/_{A}$T site for the ETS transcription factors, which may be sufficient to cause ectopic SHH expression in the anterior limb bud.[27]

In **paper III**, a novel, clinically recognizable, 17q22 microdeletion syndrome is described. The commonly deleted region of all six patients on 17q22 identified in this study is ~0.24Mb in size, encompasses two genes: *NOG* and an open reading frame gene called *C17ORF67*. The region is rich in segmental duplications. Segmental duplications comprise ~5% of the human genome and are known to mediate clinically relevant deletions, duplications, and inversions through non-allelic homologous recombination.[88] The syndrome includes intellectual disability, visual impairment, distinctive facial features such as thin border of upper lip, upslanting palpebral fissures, micrognathia, hypertelorism and NOG-related bone and joint features such as symphalangism and conductive hearing loss. Larger deletions across the region and heterozygous missense and nonsense mutations in *NOG* have previously been shown to cause symphalangism.[42] All six patients in our study had bone and joint symptoms described in the five *NOG*-related symphalangism spectrum disorders described in OMIM.[42] Mutations in *NOG*-related symphalangism spectrum disorders have not resulted in intellectual disability.[89-92] Two previously reported patients with microdeletions including *NOG* were intellectually disabled.[93,94] All patients in our study, including patient 6, have intellectual disability. However, all cases harbored deletions of several other dosage sensitive genes outside the region of overlap, making haploinsufficiency of *NOG* and C17ORF67 unlikely to be the cause of

intellectual disability in our patients. The short and broad phalanges and symphalangism of joints in digits and toes are most likely caused by haploinsufficency of *NOG*.

Patient 2 displayed one of the most interesting phenotypic and genotypic differences in paper III. She was the only one with hypogonadotrophic hypogonadism and absence of uterus. Shimizu et al.[94] have also reported a patient with 17q22 deletion and hypogonadotrophic hypogonadism. To our knowledge, there is no human phenotype related to hypogonadism or absence of uterus reported in PubMed, OMIM or Ensemble for mutations in the genes of the deleted regions that the patient reported by Shimizu *et al.* [94] and our patient 2 share. However, Orimo *et al.* [95] have shown that mice carrying a loss of function mutation in one of the genes: Trim25, also named Efp (estrogen-responsive finger protein), have underdeveloped uterus, suggesting that Efp could be involved in the normal estrogen-induced cell proliferation of uterus and the uterine swelling. The two male patients of study III (patient 2 and 6) displayed cryptorchidism and penile chordee. Their microdeletion also involved *TRIM25*, making *TRIM25* an interesting candidate gene for further studies of urogenital malformations.

In **paper IV** three previously non-described variants in the highly conserved *FGF16* gene in three unrelated cases with X-linked recessive fusion of the fourth and fifth metacarpals, MF4 (OMIM#309630) is presented. One nonsense mutation in exon 2 and two likely pathogenic variants in conserved regions in exon 1 and 2. Two previous patients with MF4 and no other complaints have been reported with *FGF16* nonsense mutations.[96] Jamsheer *et al*[96] suggest that the position of these two nonsense mutations in the last exon 3 make the transcripts likely to be stable and escape nonsense-mediated decay. Therefore, the truncated proteins could still exert some residual activity during embryogenesis, which might prevent severe heart developmental defects as reported in *Fgf16* deficient mice.[97,98] As the nonsense mutation in family 1 in our study is positioned upstream to those mutations, in exon 2, the transcript is more likely to be a subject to nonsense-mediated RNA decay. But, if the transcript escapes nonsense-mediated decay it will be truncated before the heparin-binding site of FGF16, which may contribute to the pathogenicity of the mutation.

Family 1 in **paper IV** is the only known family described in the literature where frequent cardiac manifestations have occurred among *FGF16* mutation carrying family members. One may speculate that the above described differences in location of the truncating mutation may explain the early-onset cardiac symptoms in family 1 and the absence of cardiac symptoms in those reported by Jamsheer *et al.*[96] Alternatively, different mutations may affect the level of susceptibility of an individual to develop heart disease in response to lifestyle choices or another condition, like diabetes. There is evidence in mice that the genetic background can modify the severity of the phenotype detected with *Fgf16* knockouts.[97,98] In accordance with the cardiac manifestations in the *FGF16* nonsense mutation carrying family members, 76% of 101 zebrafish embryos injected with Fgf-16 morpholinos in this study had a heart oedema, figure 13. Also, the size of the fins was approximately half the size of controls at 72-96 hours post fertilization. In a previous *fgf16* knock-down study by Nomura *et al* the fins of zebrafish are only visible as shallow domes at 72 hours post fertilization.[74]

Phenotype variability was observed among affected individuals in family 1 (figure 12). For example, the least severely affected individual only has a broad proximal fifth metacarpal bone and an overall shorter fifth digit. The more severely affected individuals have undergone surgery due to dysfunctional positions with ulnarly deviated and hypoplastic fifth digits with fusion between the fourth and fifth metacarpals. In family 2 and in the report by Habighorst *et al*, one female in each family had the MF4 phenotype.[99] A probable explanation for the presence of symptoms in heterozygous female mutation carriers may be skewed X-chromosome inactivation.

7 CONCLUSIONS

Paper I: In Stockholm County, the incidence of CULA is 21,5/10000 live births, which is similar to the figure obtained in the only other total population study published to date. Twenty-three percent have associated anomalies and 18 percent have affected family members. Some diagnose-specific observations were noted; for instance no affected relatives were recorded in the group of patients with transverse arrest and associated anomalies are rare in symbrachydactyly while the majority of patients with radial hypoplasia/aplasia had associated non-hand anomalies.

Paper II: Insertions in Zone of polarizing activity Regulating Sequence, ZRS, can add multiple limb-related transcription factor binding sites through the sequence $AGGAA^{G}/_{A}T^{27}$ and be sufficient to drive an ectopic expression in the mouse limb bud similar to the ectopic expression of *SHH* that causes preaxial polydactyly.

Paper III: The similarities in phenotype between the individuals in **paper III** represent a novel clinically recognizable 17q22 microdeletion syndrome, in part due to haploinsufficiency of *NOG*. The dissimilarities in phenotype and genotype between the patients can be used to find other disease-associated genes.

Paper IV: Mutations in *FGF16* cause X-linked recessive metacarpal 4-5 fusion and truncating nonsense mutations in *FGF16* could contribute to heart disease. Further studies are needed to highlight pathogenic mechanisms of the *FGF16* mutations, and the possible correlation between *FGF16* mutations and human heart disease needs to be explored in a larger number of patients.

8 FUTURE PERSPECTIVES

The ultimate question is if the findings obtained in this thesis matter with respect to the clinical management of the patients. We believe they do. In the first paper we have described laterality, associated anomalies and occurrence among relatives for all included CULA. This information gives good clinical support to parents and clinicians working with CULA. The information also gives good indications of which anomalies that should be focused on to describe causative genetic mechanisms in the future, since bilateral cases with associated anomalies and occurrence among relatives are more likely to be genetically caused than isolated unilateral ones. On the other hand, somatic mutations are continuously being described in developmental conditions. We therefore keep collecting tissue during pediatric hand surgery for further molecular studies after we have obtained informed consent. The main issue in doing so is to define which of the well-developed tissues that are embryological relevant and which tissue that is constitutional.

Fibroblast growth factors are essential to embryonic development, cell growth, and morphogenesis including limb bud formation and outgrowth.[13,100] However, FGF16 has not been implied in limb development before and *Fgf16* knock-out mice has been reported without any limb abnormalities by day E12.5.[98] The study by Jamsheer et al. and **paper IV** of this thesis fix *FGF16* mutations to fusion of the fourth and fifth metacarpals. Also, in the study by Jamsheer et al. expression of *Fgf16* in mice at day 12.5 was observed in the central interdigital areas of the fore- and hindlimbs.[96] Further studies are needed to rule out in what way or pathway *FGF16* acts on limb development and if other ulnar dominated CULA are caused by *FGF16* mutations.

Further genotype-phenotype evaluation should be performed in additional patients with 17q22 deletions and functional studies should be performed in order to characterize genes important for cognitive function and heart and urogenital development.

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APPENDIX 1

Incidence, relative frequency, gender distribution, affected side, occurrence among relatives, and associated non-hand anomalies in 562 children with 585 CULA. From Ekblom et al.[67] with permission from publisher

Incidence, Relative frequency, Gender, Affected side, Associated non-hand anomalies and Ocurrence among relatives in 585 CULA in 562 Children born in Stockholm, Sweden 1997-2007. IFSSH Classification.

Congenital Upper Limb Anomaly, IFSSH Classification	Number of anomalies	Incidence per 10 000 live births	Relative frequency (%)	Gender					Affected side						Associated anomalies		Ocurrence among relatives	
				Male No	Male %	Female No	Female %		Right No	Right %	Left No	Left %	Bilateral No	Bilateral %	No	%	No	%
I. Failure of formation	**103**	**3,9**	**17,6**	**69**	**67,0**	**34**	**33,0**		**36**	**35,0**	**42**	**40,8**	**25**	**24,3**	**30**	**29,1**	**10**	**9,7**
A. Transverse arrest	22	0,8	3,8	13	59,1	9	40,9		9	40,9	13	59,1	0	0,0	6	27,3	0	0,0
B. Longitudinal arrest	81	3,1	13,8	56	69,1	25	30,9		27	33,3	29	35,8	25	30,9	24	29,6	10	12,3
Radial ray deficiency	33	1,3	5,6	17	51,5	16	48,5		9	27,3	7	21,2	17	51,5	20	60,6	2	6,1
Thumb deficiency	20	0,8	3,4	8	40,0	12	60,0		6	30,0	3	15,0	11	55,0	9	45,0	2	10,0
Radius hypolastic/abscent	13	0,5	2,2	9	69,2	4	30,8		3	23,1	4	30,8	6	46,2	11	84,6	0	0,0
Ulnar ray deficiency	25	1,0	4,3	21	84,0	4	16,0		10	40,0	9	36,0	6	24,0	2	8,0	4	16,0
Central ray deficiency	23	0,9	3,9	18	78,3	5	21,7		8	34,8	13	56,5	2	8,7	2	8,7	4	17,4
Cleft hand	8	0,3	1,4	7	87,5	1	12,5		4	50,0	3	37,5	1	12,5	2	25,0	3	37,5
Symbrachydactyly	15	0,6	2,6	11	73,3	4	26,7		4	26,7	10	66,7	1	6,7	1	6,7	1	6,7
Intersegmental arrest	0	0,0	0,0															
II. Failure of differentiation	**276**	**10,5**	**47,2**	**137**	**49,6**	**139**	**50,4**		**78**	**28,3**	**83**	**30,1**	**115**	**41,7**	**47**	**17,0**	**52**	**18,8**
A. Soft tissue involvement	216	8,2	36,9	111	51,4	105	48,6		63	29,2	77	35,6	76	35,2	38	17,6	34	15,7
Sprengel deformity	5	0,2	0,9	2	40,0	3	60,0		1	20,0	4	80,0	0	0,0	4	80,0	0	0,0
Poland syndrome	4	0,2	0,7	4	100,0	0	0,0		3	75,0	1	25,0	0	0,0	1	25,0	0	0,0
Cutaneous syndactyly	36	1,4	6,2	21	58,3	15	41,7		5	13,9	6	16,7	25	69,4	16	44,4	19	52,8
Camptodactyly	40	1,5	6,8	23	57,5	17	42,5		9	22,5	13	32,5	18	45,0	8	20,0	8	20,0
Thumb in palm deformity	7	0,3	1,2	5	71,4	2	28,6		0	0,0	1	14,3	6	85,7	1	14,2	1	14,3
Dewated finger without bony deformity	4	0,2	0,7	2	50,0	2	50,0		2	50,0	1	25,0	1	25,0	0	0,0	1	25,0
Wind-blown hand	5	0,2	0,9	4	80,0	1	20,0		0	0,0	0	0,0	5	100,0	1	20,0	2	40,0
Congenital trigger digit or thumb	115	4,4	19,7	50	43,5	65	56,5		43	37,4	51	44,3	21	18,3	7	6,0	3	2,6
B. Skeletal involvement	60	2,3	10,3	26	43,3	34	56,7		15	25,0	6	10,0	39	65,0	9	15,0	18	30,0
Shoulder level, other	2	0,1	0,3	0	0,0	2	100,0		2	100,0	0	0,0	0	0,0	1	50,0	0	0,0
Proximal radio-ulnar synostosis	3	0,1	0,5	2	66,7	1	33,3		1	33,3	0	0,0	2	66,7	0	0,0	1	33,3
Synostosis of phalanges (complex syndactyly)	13	0,5	2,2	8	61,5	5	38,5		3	23,1	1	7,7	9	69,2	5	38,5	5	38,5
Symphalangia	1	0,0	0,2	1	100,0	0	0,0		0	0,0	0	0,0	1	100,0	0	0,0	1	100,0
Clinodactyly	31	1,2	5,3	10	32,3	21	67,7		7	22,6	4	12,9	20	64,5	2	6,5	8	25,8
Triphalangeal thumb	10	0,4	1,7	5	50,0	5	50,0		2	20,0	1	10,0	7	70,0	1	10,0	3	30,0
III. Duplication	**155**	**5,9**	**26,5**	**91**	**58,7**	**64**	**41,3**		**45**	**29,0**	**50**	**32,3**	**60**	**38,7**	**40**	**25,8**	**39**	**25,2**
Digit	155	5,9	26,5	91	58,7	64	41,3		45	29,0	50	32,3	60	38,7	40	25,8	39	25,2
Radial polydactyly	59	2,3	10,1	34	57,6	25	42,4		33	55,9	17	28,8	9	15,3	13	22,0	14	23,7
Central polydactyly	4	0,2	0,7	2	50,0	2	50,0		2	50,0	1	25,0	1	25,0	0	0,0	3	75,0
Ulnar polydactyly	92	3,5	15,7	55	59,8	37	40,2		10	10,9	32	34,8	50	54,3	27	29,3	22	23,9
IV. Overgrowth	**10**	**0,4**	**1,7**	**5**	**50,0**	**5**	**50,0**		**5**	**50,0**	**3**	**30,0**	**2**	**20,0**	**3**	**30,0**	**0**	**0,0**
Hemihypertrophy	7	0,3	1,2	4	57,1	3	42,9		4	57,1	3	42,9	0	0,0	2	28,6	0	0,0
Macrodactyly	3	0,1	0,5	1	33,3	2	66,7		1	33,3	0	0,0	2	66,7	1	33,3	0	0,0
V. Undergrowth	**18**	**0,7**	**3,1**	**5**	**27,8**	**13**	**72,2**		**4**	**22,2**	**6**	**33,3**	**8**	**44,4**	**5**	**27,8**	**3**	**16,7**
Whole limb	2	0,1	0,3	0	0,0	2	100,0		1	50,0	1	50,0	0	0,0	0	0,0	0	0,0
Brachymetacarpia	3	0,1	0,5	0	0,0	3	100,0		0	0,0	1	33,3	2	66,7	1	33,3	0	0,0
Brachysyndactyly	2	0,1	0,3	0	0,0	2	100,0		1	50,0	1	50,0	0	0,0	1	50,0	0	0,0
Brachydactyly	11	0,4	1,9	5	45,5	6	54,5		2	18,2	3	27,3	6	54,5	3	27,3	3	27,3
VI. Constriction ring syndrome	**9**	**0,3**	**1,5**	**2**	**22,2**	**7**	**77,8**		**1**	**11,1**	**2**	**22,2**	**6**	**66,7**	**4**	**44,4**	**0**	**0,0**
VII. Generalized abnormalities and syndromes	**14**	**0,5**	**2,4**	**7**	**50,0**	**7**	**50,0**		**0**	**0,0**	**0**	**0,0**	**14**	**100,0**	**11**	**78,6**	**0**	**0,0**
Total No. of Anomalies	**585**	**22,3**	**100**	**316**	**54,0**	**269**	**46,0**		**169**	**28,9**	**186**	**31,8**	**230**	**39,3**	**141**	**24,1**	**104**	**17,8**
Total No. of Children	**562**	**21,5**	**-**	**304**	**54,1**	**258**	**45,9**		**169**	**30,1**	**186**	**33,1**	**207**	**36,8**	**130**	**23,1**	**99**	**17,6**