# Clinical and Molecular-Genetic Studies in Esophageal Atresia

**Elisabeth Maaike de Jong**

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# Clinical and Molecular-Genetic Studies in Esophageal Atresia

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Waar vindt een mens
dat klein geluk
dat ongevraagd
en als vanzelf
het leven tot
een glimlach maakt?

**Henk Jongerius**

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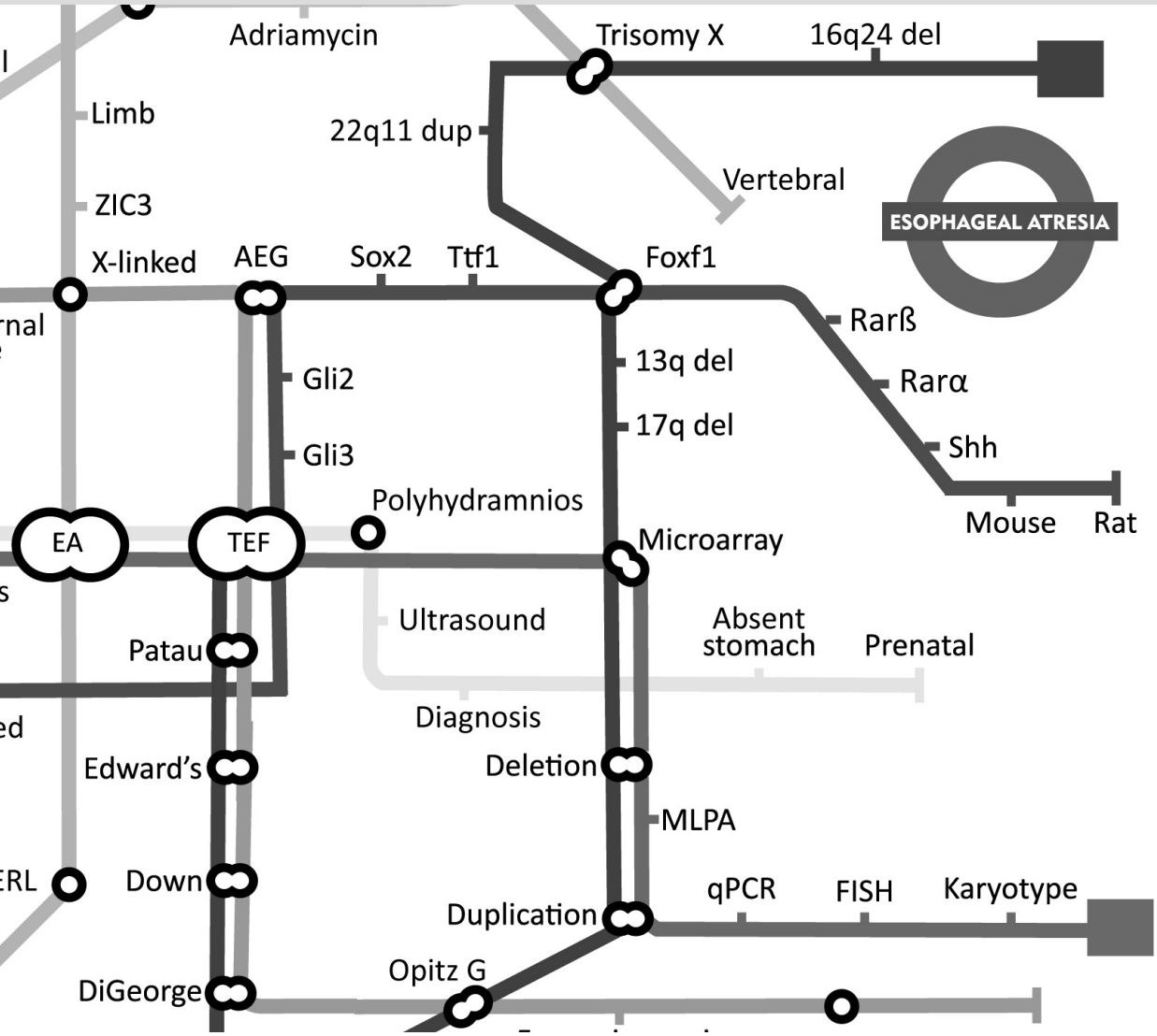
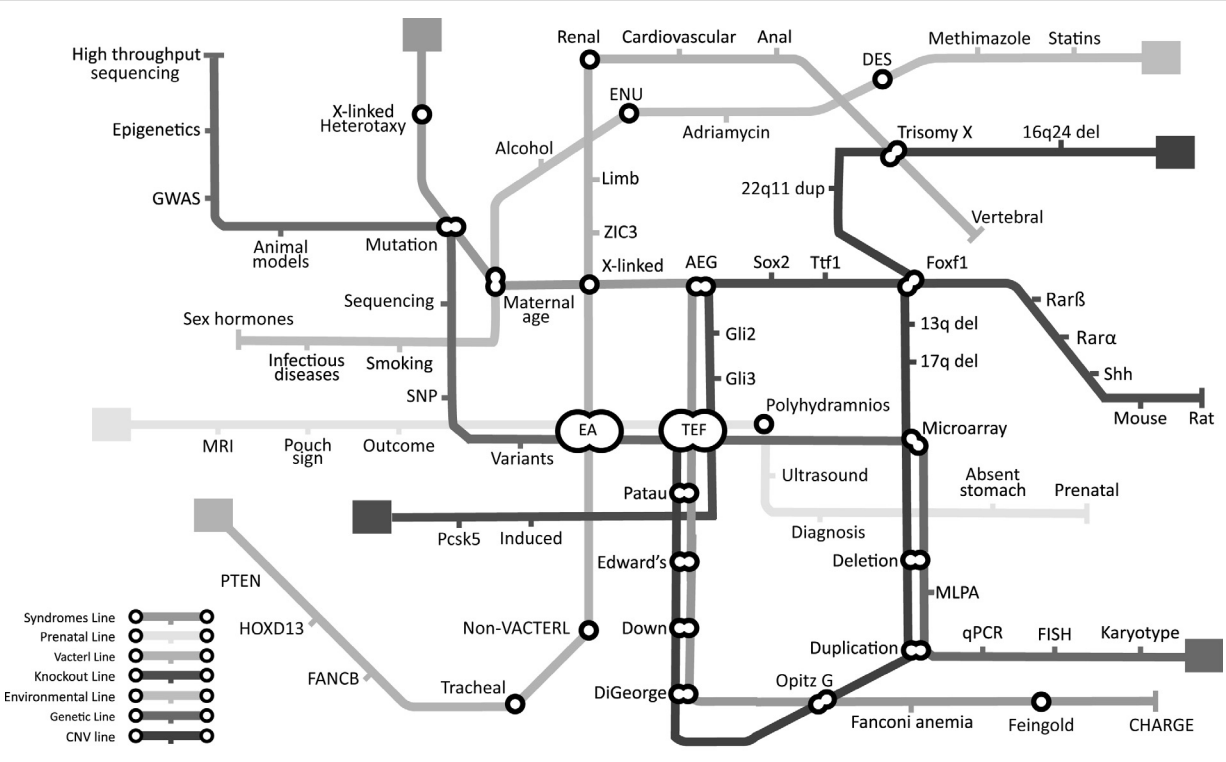
## General Introduction



# CHAPTER 1

## General Introduction

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**de Jong EM**, Eussen BH, Douben H, van Ijcken W, de Klein A (2009). Molecular cytogenetics of congenital disorders. *Molecular Diagnostics: Tools and Applications.* W. van Leeuwen and C. Vink: 139-148.

# Introduction

**E**sophageal **a**tresia (EA) and **t**rachea-**e**sophageal **f**istula (TEF) [MIM 189960] are severe developmental defects that affect one in 3,500 newborns. Slightly more boys are affected than girls[1]. In The Netherlands, approximately 70 children are born annually with this congenital malformation. It is characterized by a lack of continuity of the upper gastro-intestinal tract that is often accompanied with a persistent connection, or fistula, between the trachea and the esophagus (tracheo-esophageal fistula). Five subtypes are described by the Gross classification (Figure 1A)[2]. Subtype C, with a blind-ending proximal pouch (atresia) and a distal TEF, occurs most frequently (86%), followed by the pure proximal atresia (subtype A; 7%). As is the case with other congenital malformations, EA/TEF occurs more in twins than in singletons, although usually only one twin is affected[3]. Once a couple has one child with EA/TEF, the risk of having a second child with this anomaly goes up to 1%[4]. Over 50% of infants with EA have additional anomalies, e.g. vertebral, gastro-intestinal, urogenital, cardiovascular, renal or limb abnormalities[5-7].

EA/TEF is the result of faulty septation of the foregut. Other congenital anomalies of foregut-derived structures, such as tracheal agenesis, are also of interest. In tracheal agenesis the trachea is fully or partially absent. It occurs in one in every 50,000 births[8]. Like EA/TEF, tracheal agenesis may be part of a complex of malformations, and associations with notably cardiovascular anomalies have been reported[9,10].

### *Presentation and management*

Clinical symptoms and ultrasound signs during pregnancy could suggest the presence of EA. The presence of a polyhydramnios may suggest gastro-intestinal obstruction, but is also related to a wide variation of other anomalies[11,12]. Polyhydramnios combined with a small or invisible stomach raises the positive predictive value for EA/TEF to 39-56%[12-16]. This combination lacks specificity, however, because amniotic fluid may flow into the stomach when a TEF is present. Efforts to improve the detection rate of EA/TEF have focused on ultrasound or MRI demonstration of an esophageal pouch; i.e. a dilatation of the blind-ending upper esophageal segment[12,17-22]. Prenatal detection of EA is difficult; therefore most often EA is diagnosed postnatally.

Postnatally, EA is suspected in the presence of excessive oropharyngeal secretions, with or without respiratory difficulties, and the inability to pass a nasogastric tube, confirmed on chest radiograph. The two ends of the esophagus are usually surgically anastomosed within 24-48 hours from the diagnosis[6]. From 2002 onwards pediatric surgeons have used

a thoracoscopic approach[23]. Recent studies evaluated the safety, efficacy and outcome of this approach[24-27]. From the findings it is clear that it will become the procedure of choice, provided the surgeon is well experienced.

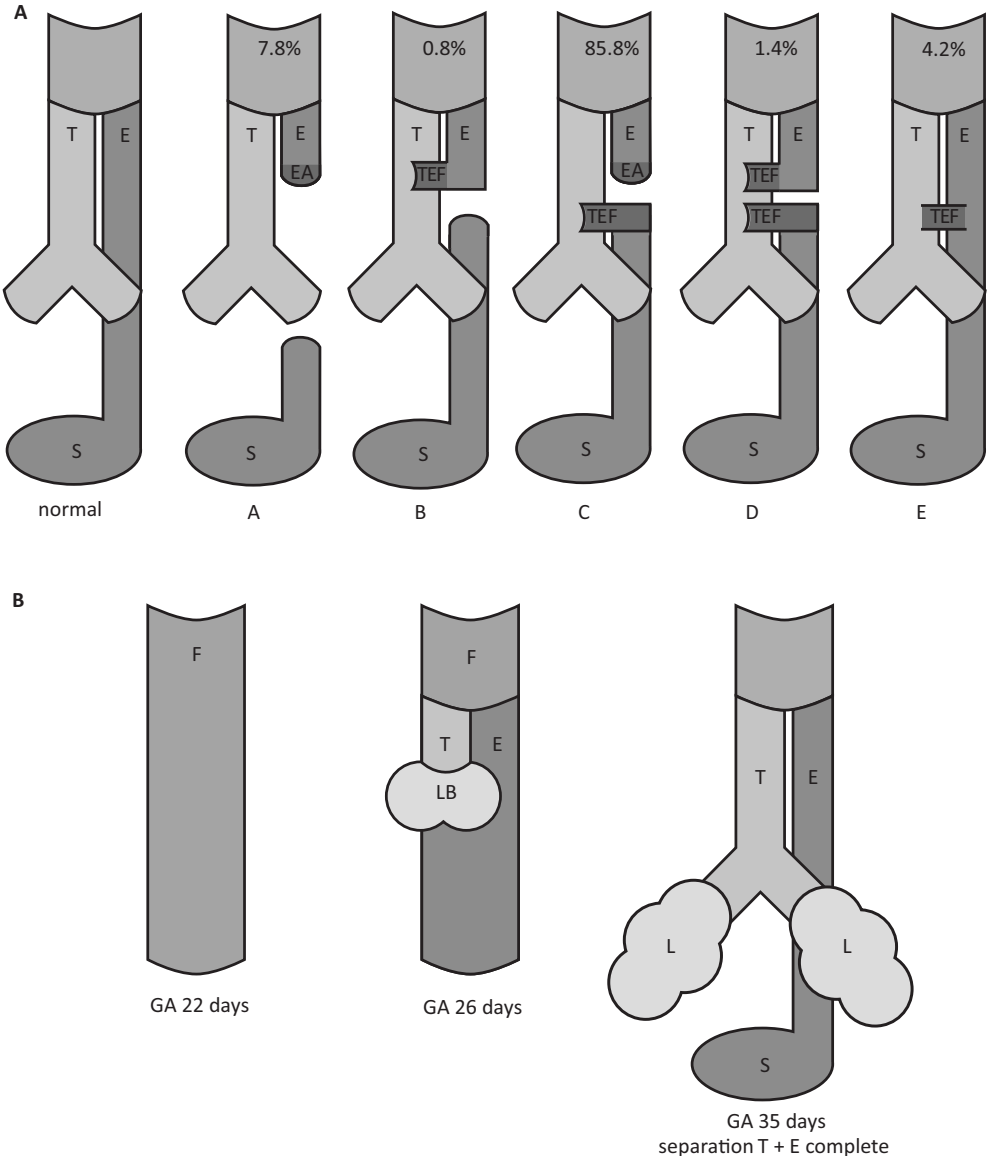


**Figure 1**

A: Classification system for EA/TEF according to Gross (1953)

B: Schematic representation of normal development of the foregut

F: foregut, L: lung, LB: lung bud, E: esophagus, T: trachea, S: stomach, GA: gestational age. *Color figures can be found in the appendix. See page 180*

Possible post-operative complications are anastomotic strictures and leaks, recurrent tracheo-esophageal fistula, gastro-esophageal reflux and sequelae of thoracotomy, such as scoliosis and thorax asymmetry. Although mortality and complication rates have decreased over the past decades, EA/TEF may still cause significant short- and long-term morbidity[6,28-34].

### *Embryology*

The trachea, esophagus and lungs are foregut-derived structures. The endodermal layer of the early embryo converts into the primitive gut tube. During the fourth week of embryonic life, the proximal foregut divides into a ventral respiratory part and a dorsal gastro-intestinal part. This process is initiated by a ventral evagination from the foregut with the two lung buds (Figure 1B). The separation of esophagus and trachea is complete at day 35 of gestation.

The process by which the trachea and esophagus are defined and separated is not well understood. The mechanisms for normal tracheo-esophageal embryogenesis have recently been reviewed, focusing on both normal and abnormal morphogenic mechanisms and controversies in the development of EA/TEF[35,36]. For example, competing theories of separation exist, e.g. fusing of lateral longitudinal tracheo-esophageal folds in a caudal to cranial direction (traditional theory)[37], folds that grow into the tracheo-esophageal space (no fusing)[38,39], or stages or epithelial proliferation and apoptosis by which the trachea and esophagus separate[40,41]. A different theory was proposed by Crisera *et al.,* studying the adriamycin rat model. They proposed that an atresia of the foregut is the primary event, leading to a trifurcation of the lung bud; the left and right branches develop into the lungs and a third branch forms the tracheo-esophageal fistula[39,42].

### *Etiology*

A plethora of genetic and environmental factors have been suggested to play an etiological role in EA/TEF, but the developmental mechanisms are far from clear (for overviews, see Felix *et al.* 2004 and 2009)[43,44]. Studying specific abnormalities in genes or genetic pathways associated with either syndromic or isolated EA/TEF is expected to be helpful[5]. In addition, knowledge about environmental factors and their possible interactions with genetic factors may provide further clues. Unraveling the etiology may be a starting point for preventive strategies and parental counseling.

## Genetic factors

From 6 to 10% of postnatally diagnosed patients with EA are also diagnosed with a chromosomal abnormality or syndrome[5]. An overview of genetic syndromes and the corresponding phenotypes is given in Table 1. Genetic abnormalities associated with EA/TEF were found in animal studies as well.

### *Human syndromes and associations*

EA/TEF is present in a number of syndromes and associations. Of these, the VACTERL (**V**ertebral, **A**nal, **C**ardiac, **T**racheo-**E**sophageal, **R**enal, **L**imb) association is the best known, seen in any of its various presentations in 10-30% of infants with EA/TEF[1,45,46]. Currently, the etiology of VACTERL association is unclear. Little is known about a possible genetic predisposition. A few studies, however, found causative mutations in the genes *FANCB, HOXD13*, *PTEN,* and *ZIC3* in some patients with VACTERL-associated anomalies combined with other major anomalies, such as hydrocephalus or heterotaxy[47-50]. However, no specific genetic locus has been found yet. Recently Stankiewicz *et al.* described deletions on the 16q24.1 locus in syndromic patients with overlapping anomalies of the VACTERL association. This locus includes the forkhead genes *FOXF1*, *FOXC2* and *FOXL.* Of these transcription factors, FOXF1 is of particular interest, because heterozygote knockout mice for this protein show EA/TEF[51].

Also, EA/TEF occasionally occurs in combinations with hemifacial microsomia, cardiac, vertebral and/or central nervous system anomalies (Goldenhar syndrome). This syndrome has a familial occurrence, but no genetic basis has been described[52-56].

Around 30-40% of patients diagnosed with Feingold syndrome have EA/TEF. Mutations and deletions of the *MYCN* gene on chromosome 2p24.1 were shown to cause this syndrome[57,58]. There is every reason, therefore, to test patients with EA/TEF and other characteristics of Feingold syndrome for variants of this gene, and to counsel them on the risk of transmitting the syndrome to offspring.

EA/TEF can be part of the spectrum of anomalies seen in Fanconi anemia: mutations in the *FANCA*, *FANCB*, *FANCC*, *FANCD1* (*BRCA2*)*, FANCD2*, and *FANCG* genes have been described in patients with EA/TEF with various combinations of defects, including microcephaly, short stature, pigment changes, and several VACTERL-associated anomalies, including heart, renal, and limb defects[47,59-64].

**Table 1: Genetic syndromes and associations involving EA/TEF**

| | OMIM | Gene(s) | Locus | Major defects§ |
|---|---|---|---|---|
| ***Single gene disorders*** | | | | |
| Feingold syndrome | 164280 | *MYCN* | 2p24.1 | Intestinal atresias, microcephaly, learning disability, CHD, limb defects, short stature |
| CHARGE syndrome | 214800 | *CHD7* | 8q12 | Coloboma, CHD, choanal atresia, GR, genito-urinary and ear anomalies/deafness |
| AEG syndrome | 206900 | *SOX2* | 3q26.3-q27 | Clinical anophthalmia, GD, mesial temporal abnormalities of the brain |
| Opitz G syndrome | 300000 | *MID1* | Xp22 | Laryngotracheo-esophageal cleft, hypertelorism, hypospadias, cleft lip/palate, CHD, AA, developmental delay |
| Fanconi anemia | 607139 | *FANCA* | 16q24.3 | Anemia, abnormal skin pigmentation, short stature, microphthalmia, microcephaly, susceptibility to cancer, CHD, limb and renal defects |
| | 227645 | *FANCC* | 9q22.3 | |
| | 605724 | *FANCD1* | 13q12.3 | |
| | 227646 | *FANCD2* | 3p25.3 | |
| | 602956 | *FANCG* | 9p13 | |
| VACTERL + hydrocephalus | 300514 | *FANCB* | Xp22.31 | VACTERL-associated defects, hydrocephalus, Arnold-Chiari malformation, cleft palate, incomplete lung lobation |
| VACTERL + hydrocephalus | 276950 | *PTEN* | 10q23.31 | VACTERL-associated defects, macrocephaly, ventriculomegaly |
| Heterotaxy X-linked | 306955 | *ZIC3* | Xq26.2 | Heterotaxy, splenic defects, CHD, long lobulation defects, renal and lumbosacral defects |
| ***Chromosomal abnormalities*** | | | | |
| Full trisomies | - | | Chromosomes 13,18,21 | Major congenital anomalies, including MR, CHD, gastro-intestinal atresias, Hirschsprung disease, dysmorphic features |
| 22q11 deletion (DiGeorge) syndrome | 188400 | *TBX1* * | 22q11.2 | CHD, cleft palate, facial dysmorphism, hypocalcemia, hypertelorism, hypospadias, thymic hypoplasia and midline defects |
| Opitz syndrome | 145410 | *TBX1* * | 22q11.2 | Hypertelorism, laryngotracheo-esophageal cleft, cleft lip/palate, GD, MR, CHD |
| 13q deletion | - | *ZIC2* * | 13q22-qter | Central nervous system malformations, intestinal atresias, GR, coloboma, genitourinary, midline and VACTERL-associated defects |
| 17q deletion | - | *RARα* * *NOG* * *TBX4* * | 17q21.3-q24.2 | MR, conductive hearing loss, impaired vision, craniofacial and skeletal defects |
| 16q24 deletion | - | *FOXF1* * | 16q24.1 | ACD, VACTERL-associated defects, urinary tract obstruction |

§: other defects: in combination with EA/TEF; *: gene(s) of interest on chromosomal locus; AA, anal atresia/imperforate anus; ACD, alveolar capillary dysplasia; AEG, Anophthalmia Esophageal-Genital; GD, genital defects, including cryptorchidism, hypospadias, genital hypoplasia; CHD, congenital heart defects; GR, growth retardation; MR, mental retardation

Deletions and mutations in the *SOX2*, *CHD7* and *MID1* genes were shown to be a cause of AEG (**A**nophthalmia-**E**sophageal-**G**enital) syndrome, CHARGE syndrome and X-linked Opitz syndrome, respectively[65-67]. Que *et al*. showed that hypomorphic *Sox2* mutant mice have EA with a distal TEF, thereby providing a link between the animal and human defects[68].

Other genes implicated in both humans and animals are *GLI3* and *FOXF1*. Mutations in the *GLI3* gene can cause Pallister-Hall syndrome, in which laryngotracheo-esophageal clefts are occasionally described[69-72]. Combined *Gli2/Gli3* knockout mice display severe foregut anomalies, as described in more detail below[73].

### *Chromosomal anomalies*

EA/TEF occurs in children with trisomy 21 (0.5-1.0%) and trisomy 18 (up to 25%)[3,74-76], but also in patients with trisomy 13 (Patau syndrome) and (mosaic) trisomy X[1,3,75,77]. The causative mechanism for the anomalies in these trisomies is unknown.

A number of patients with EA/TEF have structural chromosomal anomalies (for review, see Felix *et al.*, 2007[78]; Lurie 2007[79]). As many of those display other birth defects and/or multiple chromosomal anomalies as well, it is difficult to link a specific chromosomal defect to EA/TEF.

Deletions on several chromosomal loci – including 22q11 (DiGeorge syndrome), distal 13q, 17q21.3-q24.2, and 16q24.1 – are found occasionally[51,78,80-85]. Interestingly, some of these deleted loci include several candidate genes for EA/TEF e.g., the 17q21.3-q24.2 region including *NOG* and *TBX4*, known from animal studies to be involved in foregut development[83-85]. *TBX1* is the most important candidate gene in 22q11 deletion syndrome, but no mutations in this gene have been described in patients with EA/TEF[69,80,86,87].

Studying specific genetic anomalies involved in EA/TEF may provide valuable information about the abnormal developmental processes leading to EA/TEF. Additionally, it may give insight into more general processes involved in faulty foregut development.

### *Knockout and other mutation models*

Aside from human studies, EA/TEF can be studied in experimental animal models, usually the mouse, in which specific genes have either been mutated or deleted. If the treated mouse shows a phenotype that includes EA/TEF, the likely conclusion is that the deleted or mutated gene is probably involved in the normal development of the mouse foregut. A number of genes and pathways have been studied in mice. Although these results cannot necessarily be extrapolated to humans, they can produce valuable insights into developmental mechanisms and may provide starting points for further studies in humans.

In recent years, *Sonic hedgehog* (*Shh*) and its downstream mediators have been a major focus of animal research. Shh is a small signalling molecule involved in multiple developmental processes. In mice, *Shh* null mutants have severe foregut anomalies, including EA/TEF[88]. Deletion of the chromosomal locus that contains the *SHH* gene has been reported in only one human patient with EA/TEF. This child also had other congenital anomalies[89]. Other patients reported to have deletions of this region did not have EA/TEF or other gastro-intestinal anomalies[75,90,91]. To the best of our knowledge, there are no studies describing the systematic screening of EA/TEF patients for mutations or deletions of *SHH*. Interestingly, *MYCN*, the gene involved in Feingold syndrome, is a direct signalling target of the *Shh* pathway[92,93].

Knockout models of downstream mediators of the *Shh* signalling pathway, for example several genes of the *Gli* family, which are regulatory transcription factors, have phenotypes with EA/TEF or lung abnormalities. Reducing the *Gli3* dosage, by 50% in a *Gli2*$^{-/-}$ background gives a phenotype of EA/TEF; combined knockouts for *Gli2* and *Gli3* do not form esophagus, trachea or lungs at all[94]. *Gli3* thus provides a link between the well-described *Shh* pathway in mouse foregut development and a human defect of foregut development. Also, *Shh* inhibits the expression of the growth factor *Fgf10* in the lung, while regulating the expression of *Gli3*. *Gli3* in its turn controls the expression of *Foxf1*, a transcription factor, which induces *Fgf10* expression (for a review, see Cardoso and Lu, 2006)[95]. Lack of *Foxf1* has also been shown to cause EA/TEF in haploinsufficient mice[96], and recently a deletion of the 16q24.1 locus, including the *FOXF1* gene, was described in a patient with EA/TEF[51].

In addition, Sonic hedgehog has been shown to induce *Bmp4*, a bone morphogenetic protein (Bmp) with regulatory functions[97]. Bmp signaling is present in the anterior foregut, where the tracheal primordium originates; targeted knockouts for *Bmp4* at this site have no trachea development[98]. It also has been suggested that Hox proteins are downstream proteins in the BMP-pathway[99], and Shh induces a number of *Hox* genes during development of chick hindgut[100].

Another mouse knockout model for EA/TEF is that of the BMP-antagonist *Nog*. It results in EA/TEF in around 60% of cases[101,102]. Que *et al.* demonstrated that *Nog* transcriptional processes could explain the degree of development of the trachea, and therefore could discriminate between the subtypes of tracheal agenesis[101]. In addition, *Nog* knockout mice display lung branching defects, abnormal notochord morphogenesis, exencephaly, limb, vertebral and cartilage abnormalities and lethality at birth. This phenotype does not resemble any of the human syndromes in which EA/TEF is described. *NOG* mutations in humans have been described, but no associations with EA/TEF have been reported[101].

The abnormal notochord morphogenesis, similar to those seen in the Adriamycin rat model described below, involves a prolonged connection of the notochord to the foregut[101,102]. The notochord normally expresses *Nog,* whose product is the Noggin protein. In *Nog* null mutants the *Bmp4* gene is not inhibited, leading to unrestrained BMP4 signalling and increased intercellular adhesion. This process might play a role in the development of EA/TEF[101].

Li *et al.* offered yet another hypothesis for the foregut anomalies observed in *Nog* null mutant mice. They postulate that the imprecise and delayed detachment of the notochord from the dorsal foregut causes cells from the dorsal foregut to associate with the notochord as it separates from the foregut. This would significantly reduce the number of cells of the dorsal foregut, leading to the formation of an atresia[102].

Mutations in the *MID1* gene are a cause of X-linked Opitz syndrome in humans, in which TEF has been described[67]. In the establishment of the right-left axis in chickens, *Mid1*, encoding a protein involved in cell proliferation and development, has been shown to positively influence *Bmp4* expression and to negatively influence *Shh* expression. The role of human *MID1* expression in the patterning of the left-right axis is still unclear[103].

Vitamin A has long been known as crucial for normal development. To illustrate this we refer to the finding that pregnant rats kept on a vitamin A deficient diet gave birth to pups with abnormal lung morphogenesis[104]. Vitamin A is metabolized into retinoic acid that during foregut development binds to one of its receptors and thereby influences the expression of *Hox* genes. An example is *Hoxa5*, which encodes a homeotic protein that binds to DNA in the lungs[105]. Knockout mice for different combinations of subtypes of the retinoic acid receptors tend to display abnormal morphology of trachea and esophagus, similar to that observed in vitamin A deficient pups[106,107]. Some knockout mice for the *Hoxa5* gene display tracheal occlusion as well as abnormal lung and respiratory tract development[108]. A proportion of knockout mice for another *Hox* gene, *Hoxc4*, have a phenotype that includes EA/TEF and vertebral anomalies. However, as these mice also showed affected expression of other *Hox* genes, the phenotype cannot yet be conclusively ascribed to *Hoxc4*[109].

Various other genes, such as *Thyroid Transcription Factor-1* (*Ttf-1*), are involved in foregut development and, when knocked out in experimental animals, often cause EA/TEF combined with severe anomalies of the respiratory system[110].

Another gene, *Sox2*, has recently elicited interest in both humans and animal research. The majority of hypomorphic *Sox2* mutant mice display EA/TEF[68]. In humans, mutations and deletions of *SOX2* can cause AEG-syndrome, which includes EA/TEF[65,68]. Hypomorphic *Sox2* mice also display severe eye anomalies, similar to those present in AEG-syndrome[111].

Interestingly, in the absence of Sox2 expression, the foregut tube displayed a 'tracheal phenotype', with upregulated expression of *Ttf-1*. In contrast, in *Ttf-1*$^{-/-}$ mice, the foregut showed increased *Sox2* expression, suggestive of reciprocal regulation between *Sox2* and *Ttf-1* during early foregut development[68].

A single point mutation in the *Pcsk5* gene caused a phenotype with characteristics of the VACTERL association, caudal regression syndrome and Currarino syndrome, in a mouse model using the mutagen ENU (Ethylnitrosourea)[112]. *Pcsk5* is a proprotein convertase that mediates posttranslational processing for several integrin alpha subunits. Mutations prevent its export from the endoplasmatic reticulum and destroy its proprotein convertase activity. All *Pcsk5* mutant mice had abnormal tracheo-esophageal separation. Additionally, there were pulmonary, anorectal, cardiac, skeletal, limb and renal malformations, and pre-sacral mass. The *Pcsk5* mutation resulted in abnormal expression of several *Hox* genes, including genes necessary for caudal development. Mutations in *PCSK5* were also found in human patients with VACTERL association, whose defects included tracheo-esophageal anomalies[112]. However, the mutations were inherited from a phenotypically normal parent, so their exact etiological role remains unclear.

Except for *Sox2*, *Pcsk5, Foxf1, Noggin* and *Shh*, mutations or deletions of other genes that cause EA/TEF in knockout mice have been described in humans. However, the fact that none of these patients had an esophageal anomaly, again shows that translating animal data to human malformations is problematic[113-115] (for overview: Figure 2).

## Environmental factors

### *Teratogenic models*

The Adriamycin rat model, which has also been adapted to the mouse, is a teratogenic model of EA/TEF[116,117]. Adriamycin is a glycosidic anthracyclin antibiotic used in chemotherapy. When given to pregnant rats during a specific time window in pregnancy, it produces EA/TEF in the offspring[116].

In Adriamycin-treated rats that developed EA/TEF the notochord had an abnormal position and remained attached to the foregut longer than usual, similarly to the situation described in the *Nog* knockout mouse by Li *et al.* and Que *et al.*[101,102]. In addition, the notochord showed abnormal ventral branching[118-120]. Qi *et al.* speculated that the abnormal position of the notochord may cause traction on the foregut, thus leading to occlusion or

even complete interruption of its lumen. Separation of the notochord from the foregut may therefore be a prerequisite for normal foregut development[118].

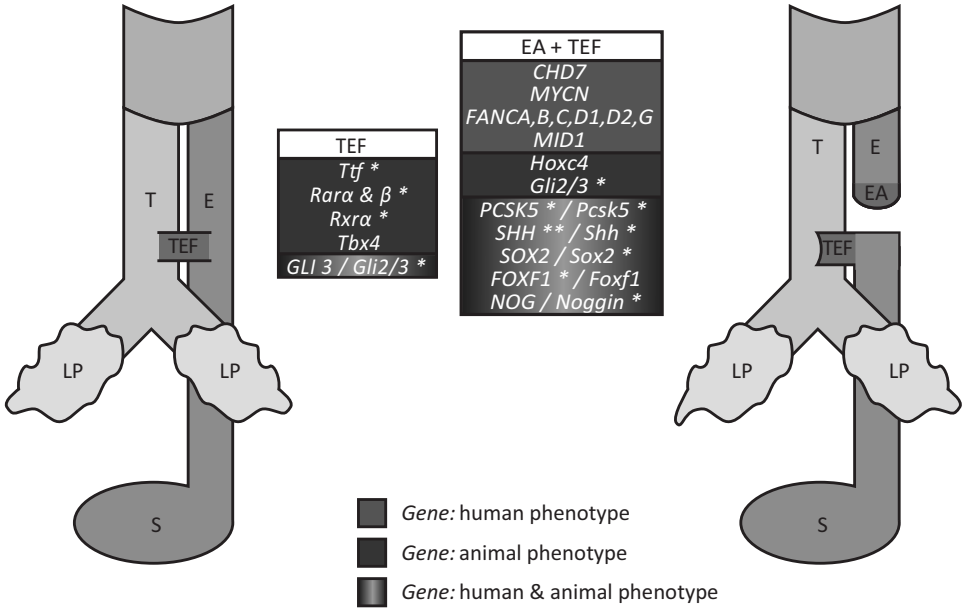


**Figure 2: Schematic representation of abnormal development of the foregut and genes involved in abnormal phenotypes**

Genes involved in abnormal development of the foregut, resulting in EA + TEF, F: foregut, L: lung, LB: lung bud, E: esophagus, T: trachea, S: stomach, TEF: tracheo-esophageal fistula, EA: esophageal atresia, LP: abnormal lung phenotype, * = lung phenotype present, ** = deletion in a single case. *Color figures can be found in the appendix. See page 180.*

The studies in the Adriamycin rat model have relevance to the teratology and genetics of EA/TEF and the VACTERL spectrum. Knockout mice for the *Shh* gene and for its downstream mediators, *Gli2* and *Gli3*, display a range of abnormalities consistent with the VACTERL spectrum[121,122]. The same pattern of anomalies is observed in rats exposed to Adriamycin[123,124]. This observation led to studies into the expression of Shh and its downstream mediators in the Adriamycin model[125].

One of the roles of the notochord in the development of the foregut is to provide a source of signaling activity[126]. Like the *Nog* knockout model described above, the abnormally close position of the notochord to the foregut in the Adriamycin-exposed rats may lead to abnormal signaling of Shh, which acts in a dose-dependent way, from the notochord to the

foregut[125,126]. Shh signalling is normally down-regulated when the notochord separates from the foregut and this is an essential prerequisite for normal tracheo-esophageal separation. In Adriamycin-exposed rats it remains high due to the close proximity of the two structures, and thus thwarts normal development of trachea and/or esophagus[125,126].

However, as EA/TEF is also found in Adriamycin-treated rats with a normal notochord and as not all Adriamycin-rats with a malformed notochord develop EA/TEF, the exact nature of the association between these two anomalies is not entirely clear[127,128].

Although no association between Adriamycin or related substances and EA/TEF has been reported in humans[129,130], this animal model is still of great value for studies into the embryology, morphology, histology and genetics of EA/TEF. For it produces EA/TEF in a manner corresponding to that seen in the commonest type in humans, that is, a proximal atresia with a distal fistula. The model's disadvantage is that Adriamycin works by interfering with DNA replication and by inhibiting DNA and RNA synthesis[131]. As such, it will not only affect the esophagus. The offspring of rats treated with Adriamycin indeed display other anomalies besides EA/TEF. Most of these are anomalies of the VACTERL-spectrum, but bladder anomalies have also been described[124]. The effects of Adriamycin exposure on the levels of expression of genes and proteins are not known.

As was mentioned above, Ethylnitrosourea, an alkylating and mutagenic agent, induces a recessive mouse mutation with a phenotype that includes abnormal tracheo-esophageal septation and VACTERL-associated anomalies[112].

### *Other environmental factors*

Various environmental factors have been suggested to be a risk for the development of tracheo-esophageal anomalies, including exposure to methimazole, statins, alcohol, smoking or exogenous sex hormones, maternal phenylketonuria, infectious disease, or work in agriculture or horticulture[132-144]. Observations in insulin-dependent diabetic mothers suggest that first trimester exposure to maternal diabetes is associated with the development of congenital anomalies, including EA/TEF and VACTERL-associated anomalies[145]. Studies from the EUROCAT birth registry network found that older mothers are at significantly greater risk of having a child with EA[146].

Previous studies implicated that maternal *in utero* exposure to diethylstilbestrol (DES) might play a role in the etiology of EA/TEF in the offspring[143]. Several hypotheses for the mechanisms of such a transgenerational effect have been proposed. These include (epi) genetic changes in the oocytes of the mother during her own fetal development, which would then become apparent in the next generation; disruption of the cell cycle of the oocytes,

making them more vulnerable to detrimental exposures; and (epi)genetic changes in the somatic cells of the exposed mothers, resulting in an altered environment that could affect their embryos[147].

So far, no specific environmental risk factor has consistently been identified, although EA/TEF is usually considered part of methimazole embryopathy.

## Molecular cytogenetics of congenital anomalies

*Introduction*

Diagnostic-genetic approaches have been applied in patients with congenital anomalies since the discovery of chromosomes. For more than 50 years, karyotyping has been a successful tool to identify chromosome aberrations in mental retardation, fetal development abnormalities, reproductive failure, and congenital malformations. It could reliably identify numerical chromosome changes and structural chromosomal rearrangements such as deletions, duplications, translocations or inversions. However, karyotyping using G-banding is limited to 5-10 Mb of resolution; the size of a band roughly depends on the availability of metaphases and the quality of the chromosome preparations. Sophisticated molecular cytogenetic techniques implemented in the last decade nevertheless allowed indentifying chromosomal aberrations smaller than 5 Mb. As an additional advantage, these new techniques use DNA as starting material. Thus there is no need for cell cultures and stored $N_2$ frozen biopsies can be used for analysis.

*Karyotyping*

One of the prerequisites of conventional chromosome analysis is the availability of metaphases. Usually blood or tissue specimens are cultured to obtain dividing cells and metaphases. Peripheral blood samples are cultured for 3-4 days but fetal samples or skin biopsies, for example, must be cultured for 1-2 weeks to obtain an adequate number of dividing cells for metaphase analysis. Synchronization techniques using thymidine or colchicine to stop the cell cycle in a particular phase to yield a larger number of metaphases. The cultured cells are fixed in methanol/acetic acid and spotted on a microscopic slide under controlled humidity conditions. In the 1950s and 1960s, the pre-banding era, numerical chromosome anomalies – e.g. trisomy 21 in Down syndrome, and aneuploidy of sex chromosomes in Turner syndrome or Klinefelter syndrome – were described as well as the first structural anomaly in cancer: the Ph (Philadelphia) chromosome, a shorter chromosome 22q, detected specific in blood and

bone marrow of patients with chronic myeloid leukaemia[148]. In the 1970s the application of chromosome banding techniques yielded many structural chromosome aberrations, such as translocations, inversions, deletions, and duplications. All these aberrations can be accurately described using the ISCN standards[149].

The G-banding technique (trypsin-Giemsa staining) became most accepted worldwide (see Figure 3). This method routinely achieves a resolution of 500-800 bands and enables to distinguish deletions or duplications larger than 5-10 Mb. In specific cases, more subtle aberrations could be recognized with high-resolution banding (>1,000 bands) using chromosomes in pro-metaphase[150]. For example, small deletions on specific chromosomes in patients suffering from Prader Willi/Angelman syndrome or velocardiofacial syndrome. Despite ongoing developments in the field of molecular cytogenetics, standard karyotyping is still the best suitable method to detect balanced anomalies such as reciprocal translocations and inversions[151].

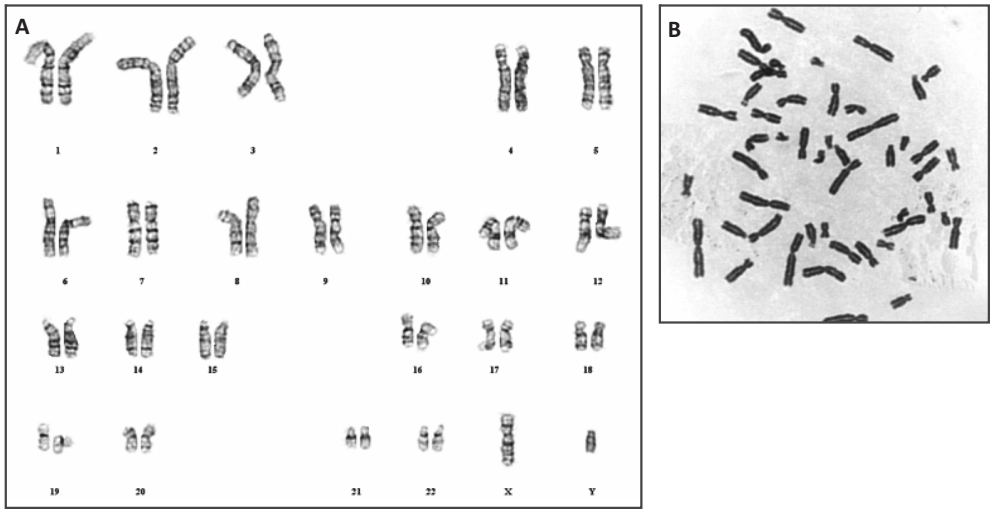


**Figure 3: Karyotype of GTG-banded chromosomes**

A Karyogram of Giemsa-trypsine G-banded chromosomes.

B The original non-banded metaphase spread is visible.

## *Fluorescent in situ hybridisation (FISH)*

FISH is a molecular cytogenetic technique in which fluorescently labeled DNA probes are hybridized to metaphase spreads or interphase nuclei[152]. A variety of probe types is available to detect aneuploidy and chromosome rearrangements. Particularly, locus-specific probes can

detect gene copy-number changes that are generally missed by classical karyotyping. This is helpful in individuals clinically diagnosed with a specific microdeletion syndrome[153,154]. FISH is also useful to detect acquired chromosomal abnormalities in tumor samples and can be used as a prognostic marker and predict response to treatment[155,156].

In a cytogenetic diagnostics setting, FISH is routinely used to confirm the clinical diagnosis of a wide variety of congenital conditions in individuals with developmental delay and mental retardation. The recurrent deletions or duplications found are 1-5 Mb large and are often flanked by highly repetitive sequence blocks facilitating the rearrangement of the chromosomal region. More than 200 of these microdeletion or microduplication syndromes have been identified. For most recurrent deletions or duplications a combination of 2-4 FISH probes[157] is sufficient to detect the anomaly with high efficiency. In a diagnostic setting commercially available FISH probes are used to detect these anomalies (Figure 4A). The wider application of genome wide screening techniques has led to the recognition of more recurrent aberrations[158-160].

To detect chromosomal imbalances at the telomeres, a specific set of sub-telomere region-specific FISH probes has been developed to simultaneously explore the entire sub-telomeric chromosomal regions in humans. These telomere aberrations are often the result of a balanced translocation in one of the parents and over 5% of the patients with idiopathic mental retardation harbor such an anomaly[161,162]. Alternatively, chromosome paints covering a whole chromosome can be used to confirm the unbalanced chromosomal translocation and to visualize a balanced translocation in the parent (Figure 4B).

FISH has several limitations. The commonly used probes (100-150 kb) are relatively large and fail to detect small deletions. Besides, no more than three to five DNA probes can be used in one experiment, thus limiting the number of targets assayed. However, specific labeling strategies allow the simultaneous visualization of all 24 human chromosomes in a single hybridization. Each chromosome is visualized in a different color, often generated by the computer program. These genome wide assays – such as Multiplex FISH (M-FISH)[163] or spectral karyotyping (SKY)[164] – are predominantly used to identify complex structural chromosomal rearrangements and marker chromosomes in prenatal and postnatal patients. Using micro-dissection of a few chromosomal bands, FISH probes combined with the above mentioned labeling strategies generate a multi-color chromosome paint. This allows easy detection and identification of complex inter-chromosomal aberrations (Figure 4C).

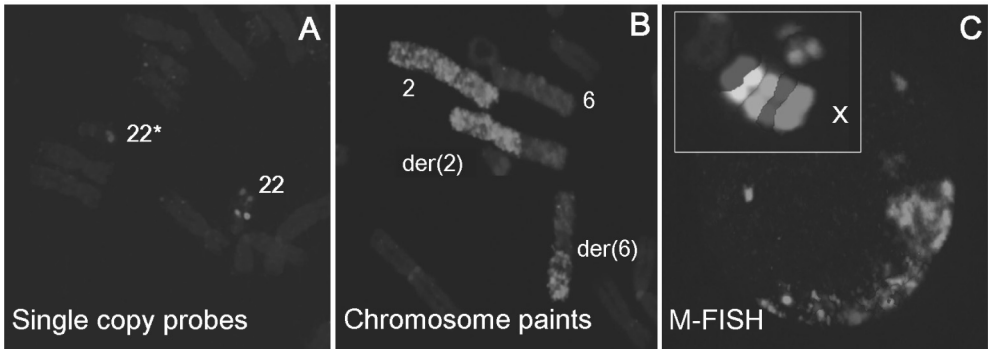


**Figure 4: Fluorescent in situ hybridisation (FISH) with different probe types**

A Single copy probes were used for 22qter (red signal) and 22q11(green signal). Note the deletion of the 22q11 locus on chromosome 22*.

B Chromosome paints specific for chromosome 2 (green signal) and chromosome 6 (red signal) were used. Derivative chromosomes of the balanced t(2;6) translocation, der(2) and der(6) are indicated.

C M-FISH probes for chromosome X. *Color figures can be found in the appendix. See page 181*

### *Quantitative methods: MLPA and qPCR*

Multiplex ligation-dependent probe amplification (MLPA), and (real-time) quantitative PCR (qPCR) have been developed to detect gene dosage alterations in a variety of prenatal and postnatal disorders. MLPA is a PCR variation that permits the amplification of multiple targets with only a single primer pair[165]. Each probe consists of a pair of primers that upon hybridization to a target sequence are subsequently ligated into a complete target sequence. The advantage of splitting the target probe is that only the ligated target sequence and not the original sample DNA is amplified. One of the MLPA primer pair is tagged with a DNA sequence of predefined length, known as the stuffer sequence. During electrophoresis, the stuffer sequence ensures that a given amplicon occurs at a known position along the gel. Different stuffer-sequence-lengths are therefore used to separate the various target amplicons. This avoids the resolution limitations of multiplex PCR. The probes are also tagged with a fluorescent label so that the intensity of the measured signal is related to the quantity of the target amplicon. Specific software programs display the quantitative results. This method is particularly suitable to detect aneuploidy and unbalanced translocation and is widely used in prenatal diagnostics[166]**.** The efficacy of this approach was demonstrated by Ahn *et al.*[167], who validated this method in a large series of over 450 patients and demonstrated its high efficiency in medium-throughput screening for subtelomere imbalances.

### *Comparative genomic hybridization*

Comparative genomic hybridization (CGH) is a technique in which differentially fluorescently labeled patient and reference genomic DNA are hybridized on metaphase chromosome slides. For each chromosome, a ratio value of the fluorescence intensity is generated from five to twenty metaphases. Thus, differential hybridization signals allow the detection of unbalanced gains and losses of chromosomal material across the whole genome[168,169]. CGH has been useful for the analysis of chromosomal imbalances in solid tumors and congenital anomalies. This technique allows the detection of unbalanced translocations, which are not detectable with standard karyotyping[170-172]. However, CGH is limited by a resolution of 3-10 Mb and aberrations with a smaller size will not be detected.

### *High density microarrays*

Introduced in 2001, microarray-based CGH techniques have changed the field of clinical cytogenetics[173]. Array CGH was initially developed to detect copy number changes in tumor samples; today it is widely used to study neuro-developmental disorders and congenital malformations with respect to genomic gains and losses[174-176]. The rapid development of different platforms with high resolutions has increased our knowledge on deletions and duplications, microdeletion and microduplication syndromes, and common variation in the human genome[177-180].

The array CGH technique is based on differentially labeled test (patient) and reference (control) genomic DNA simultaneously hybridized to a glass slide with targeted clones. After co-hybridization the intensities of the fluorescent dyes are scanned and measured. If the intensities on one spot are equal, the amounts of reference and test DNA are balanced. Differing intensities point at loss or gain of DNA, i.e. a deletion or duplication of a chromosomal region, respectively (Figure 5). The target sequences on the microarrays can be a variety of DNA substrates, e.g. bacterial artificial chromosomes (BACs), oligonucleotides or cDNAs. The resolution of the array is determined by the size of the cloned DNA targets and the distance between the targets on the genomic maps[181,182]. Initially, simple BAC-arrays with 3,500 FISH validated BACs and resolutions of 1 Mb were used[183]. As the technique developed, tiling path arrays with > 35,000 BAC clones and a resolution up to 100 kb were achieved[184]. Higher resolutions could be reached with 30-60bp oligo-nucleotide arrays and better coverage of the genome was established as well. No less than 1-3 x $10^6$ oligomers can now be spotted on an array for a whole genome screening.

Single nucleotide polymorphism (SNP) arrays were developed to detect genome wide associations (GWA) of a specific polymorphism in different disease cohorts. However, when

the intensity of the SNPs is incorporated in the analysis, these arrays can also be used to detect loss or gain of genomic DNA in a single individual.

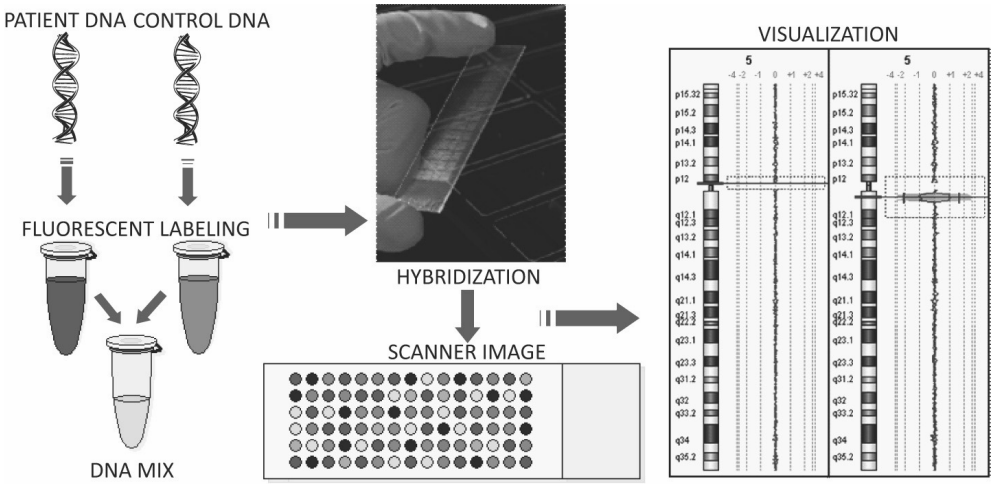


**Figure 5: Schematic representation of the microarray technology**
Genomic DNA of patient and control are both labeled with a different fluorescent dye. These are co-hybridized on a glass slide with targeted clones. The signal of hybridization is detected with a scanner. The ratio between control and patient DNA for each clone is plotted to visualize copy number variations, as indicated by a deviation from the normal log2 ratio of zero. In the panel on the right, a normal and an aberrant chromosome 5 with a 5q11 deletion detected with an Agilent® 105K Oligo-array, visualized by the Agilent® CGH Analytics Software. *Color figures can be found in the appendix. See page 181*

With up to 6 million SNPs spotted on an array, a theoretical resolution of 6kb can be established[182]. Besides copy number variations SNP platforms can also detect loss of heterozygosity and uniparental disomy, which are of interest in patients with congenital anomalies[184]. The four largest commercial microarray platforms are the oligo-array platforms from Agilent® and NimbleGen Systems® and the SNP-platforms of Affymetrix® and Illumina®.

Different logarithms and analysing tools are used to process the mountains of data. Examples of these tools are the Agilent® DNA Analytics Software and the Affymetrix® Genotyping Console™. Figure 5 illustrates the Agilent® software visualization of a normal and deleted chromosome 5q11.2.

For diagnostic screening some of these platforms have developed targeted arrays. Apart from genome wide coverage, these also include a higher density for the common micro-

deletion syndromes. The validity of this technique, an important aspect in a diagnostic setting, is still being debated.

The resolution of the technique will determine the number of aberrations that can be detected. In general 5-10 copy number variations (CNVs) are detected in a single individual. Most of these are non-pathogenic CNVs, seen in other persons as well. Common variations are collected in central databases, such as the '*Database of Genomic Variants*' (http://projects.tcag.ca/variation)[185] and the 'Copy Number Variation project' at the Children's Hospital of Philadelphia (CHOP)[186]. Some CNVs are recurrent in a given population and can serve as a signature for a specific ethnic origin. In addition, it is important to check possible pathogenic CNVs in an in-house run control set of healthy individuals (local reference set), to distinguish them from non-pathogenic CNVs. This will eliminate the common variations[178].

The quality of the DNA also determines the resolution of the technique. Hybridization with minute amounts of DNA or low quality DNA, e.g. from formalin-fixed-paraffin-embedded (FFPE) tissues results in an increase of the background and therefore requires adjustments in the analysis settings. New labeling methods and protocols to process DNA of FFPE samples enlarge the groups of patients that can be investigated with these techniques[187].

The different array platforms have a comparable coverage at comparable costs. Still, the possibility to design your own array content (targeted arrays) can be an advantage for specific diagnostic or research demands.

Experiments take about five days, mainly due to the hybridization time. As the costs are going down and process automation is expected to advance, the microarray technology will develop into a robust diagnostic tool in the field of molecular cytogenetics.

### *Future prospects*

With the development of multiplex-qPCR and microarray techniques, clinicians and researchers have powerful tools to identify genetic changes underlying congenital disorders. Although karyotyping and FISH are useful for the detection of balanced anomalies, array-CGH has proven to be instrumental in the identification of disease-related chromosomal anomalies[188,189] (see Figure 6).

Both clinical and genomic data are collected in large international databases such as DECIPHER (https://decipher.sanger.ac.uk) and ECARUCA (www.ecaruca.net). The detection of rare aberrations with concomitant clinical features has lead to the recognition of new genetic syndromes. Although this process will continue, new techniques, such as exome and high-throughput sequencing[190,191] or 4C analysis[192], are gaining interest. Although still very

expensive, these strategies are promising because they provide a solution to detect balanced translocations and inversions.

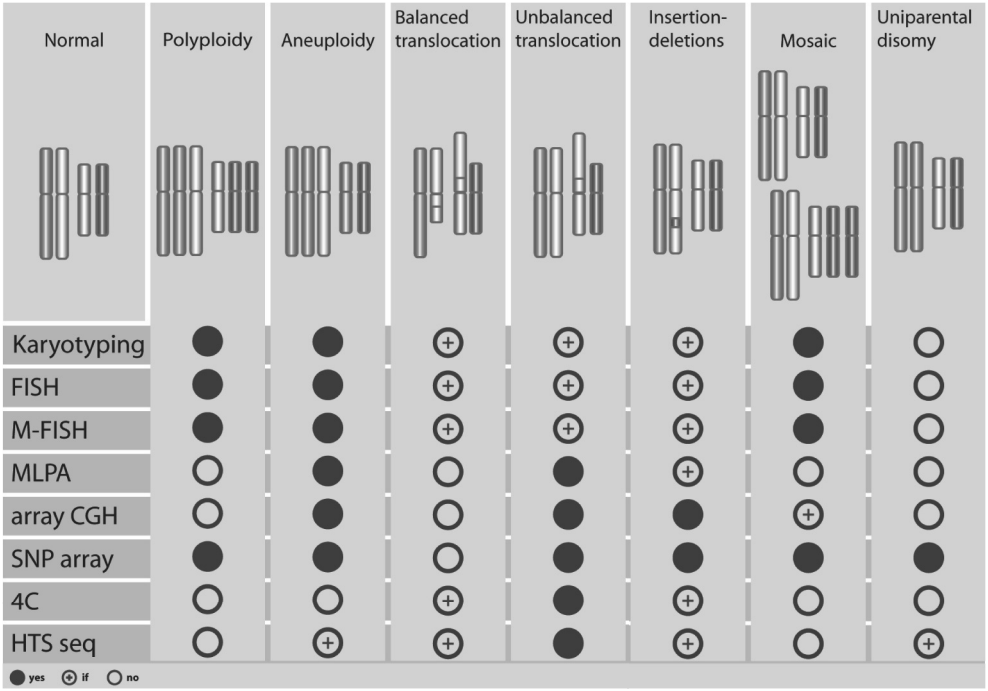


**Figure 6: Resolution of the different molecular (cyto)genetic techniques**
Schematic presentation of the possibilities and suitability of a specific technique to detect the chromosomal aberration (adapted from Speicher *et al.*[188]). At the top, the most common anomalies are depicted. Two chromosome pairs are shown in red and blue. To distinguish between the two parental copies, one of the chromosomes is darker shaded. Filled circles indicate that the technique is suitable to detect the aberration and open circles indicate that the technique is not suitable. Open circle with a '+' show that some of the anomalies can be detected with the technique, depending on probe content or percentage mosaicism. UPD: Uniparental disomy; 4C: chromosome conformation capture-on-chip; HTP seq: high-throughput sequencing. *Color figures can be found in the appendix. See page 181*

## Aim and outline of the thesis

In the field of etiological factors in EA/TEF there are many 'gaps to bridge'. The contributions of different etiological factors may vary by patient. This thesis in particular explores the genotype-phenotype relationship of EA/TEF and associated anomalies. The studies therefore focus on the phenotypic description of EA/TEF patients with an associated anomaly (notably VACTERL),

and on the use of genetic-diagnostic approaches in unraveling new genetic etiological factors involved.

We used a combined genetic approach in the search for copy number variations (deletions/duplications). This included karyotyping, screening with Multiplex ligation-dependent probe amplification (MLPA), and a genome-wide approach with microarrays.

The study cohort consisted of 321 EA/TEF patients treated at the Erasmus MC – Sophia Children's Hospital since 1988. Recently also patients from the Pediatric Surgical Center Amsterdam (VUmc/AMC) are included in the study. Clinical data, such as prenatal variables, patient characteristics, associated malformations and family pedigrees were collected and stored in the Sophia MCA database. We carefully phenotyped the patients, with a special interest in patients with VACTERL associated anomalies (Chapter 3). In addition, ten patients with tracheal agenesis were recorded in our database. Stored DNA, cell lines and tissue of the patients and parental DNA was analyzed.

Table 2 gives an overview of general characteristics and genetic data of the Erasmus MC-cohort. In eight of the 321 patients, EA/TEF had been diagnosed at autopsy after a termination of pregnancy, at the Pathology Department. Over 55% of patients had one other major congenital anomaly. Eighty-one of them could be typed as VACTERL associated, with at least two other anomalies of the VACTERL spectrum besides EA/TEF. In 37 patients a chromosomal abnormality or a single gene disorder was causative to the EA/TEF phenotype. On average, in one in every 10 patients a defined syndrome was present, which is in line with the literature. Many of the known genetic syndromes were seen in this cohort, including all full trisomies (Down Syndrome, Edwards syndrome, Patau syndrome), single gene disorders (such as CHARGE syndrome, Feingold syndrome, Opitz syndrome, and Fanconi anemia), and some less frequent syndromes (such as Holt-Oram syndrome and Townes Brocks syndrome). The distribution of syndromes is according to the literature[5,193].

From 1998 onwards, the postnatal follow-up of patients with congenital anomalies was standardized[34]. This program includes regular examinations by specialists and further genetic evaluation by a clinical geneticist. All patients from 1998 onwards were karyotyped.

Part I introduces the subject and gives an overview of the current state of knowledge on the etiology of EA/TEF. In addition, it discusses the molecular-genetic approaches for congenital anomalies used in daily practice (Chapter 1).

Part II presents the results of clinical studies in this cohort, focusing on the phenotypes of patients who also showed VACTERL association and additional congenital malformations (Chapter 3). Furthermore we studied the relation between time of diagnosis (pre- or postnatal) and outcome, specifically in patients who also showed VACTERL association (Chapter 2).

**Table 2: Characteristics and syndromes found in the Erasmus MC – Sophia cohort of EA/TEF patients**

| Characteristics | | No of cases | Syndromes in cohort | No of cases |
|---|---|---|---|---|
| *Database of EA/TEF patients since 1988* | | *321* | *Aberrant karyotypes* | |
| | EA with TEF | 274 | Down Syndrome (47,XX/XY,+21) | 6 |
| | EA type A ſ | 27 | Edwards syndrome (47,XX/XY,+18) | 7 |
| | Solely TEF | 20 | Patau syndrome (47,XY,+13) | 1 |
| | | | 47,XXX | 3 |
| Termination of pregnancy | | 8 | 46,XY,t(3:15)(q28;q22)[4]/46,XY[6] | 1 |
| Neonatal death * | | 33 | 46,XY,t(5;10)(q13;q23)pat | 1 |
| Isolated cases | | 186 | *Syndromes* | |
| Associated cases | | 135 | 22q11 deletion syndrome | 1 |
| | VACTERL associated | 81 | CHARGE syndrome | 7 |
| Tracheal agenesis | | 10 | Goldenhar Syndrome | 1 |
| | | | Opitz G syndrome | 3 |
| Karyotypes 46,XX / 46,XY | | 250 | Townes Brockes syndrome | 1 |
| Aberrant karyotypes | | 19 | Holt-Oram syndrome | 1 |
| DNA available | | 190 | Feingold syndrome | 1 |
| | | | Fanconia Anaemia | 1 |
| | | | Klippel-Feil syndrome | 1 |
| | | | X-linked heterotaxy | 1 |

* Neonatal death: death of a liveborn infant within the first 28 days of life
ſ, according to the Gross Classification

Part III describes the findings of genetic studies in patients with EA/TEF in the search for CNVs and specific genetic syndromes (Chapters 4-8). Chapter 4 describes a familial case of combined esophageal and anal atresia and the occurrence of the 22q11 microduplication syndrome. The rare finding of several aberrations on chromosome X in our cohort is presented in Chapter 5. A deletion on chromosomal locus 5q11.2 in a patient with tracheal agenesis is described in Chapter 6. Chapter 7 describes the application of high-resolution single-nucleotide polymorphism microarrays to identify novel and inherited genomic aberrations. In addition, a mutation in the *ZIC3* gene is described in a patient with EA/TEF and heterotaxy and VACTERL associated malformations (Chapter 8).

In Part IV the results of this thesis are discussed and an overall summary is provided (Chapter 9).

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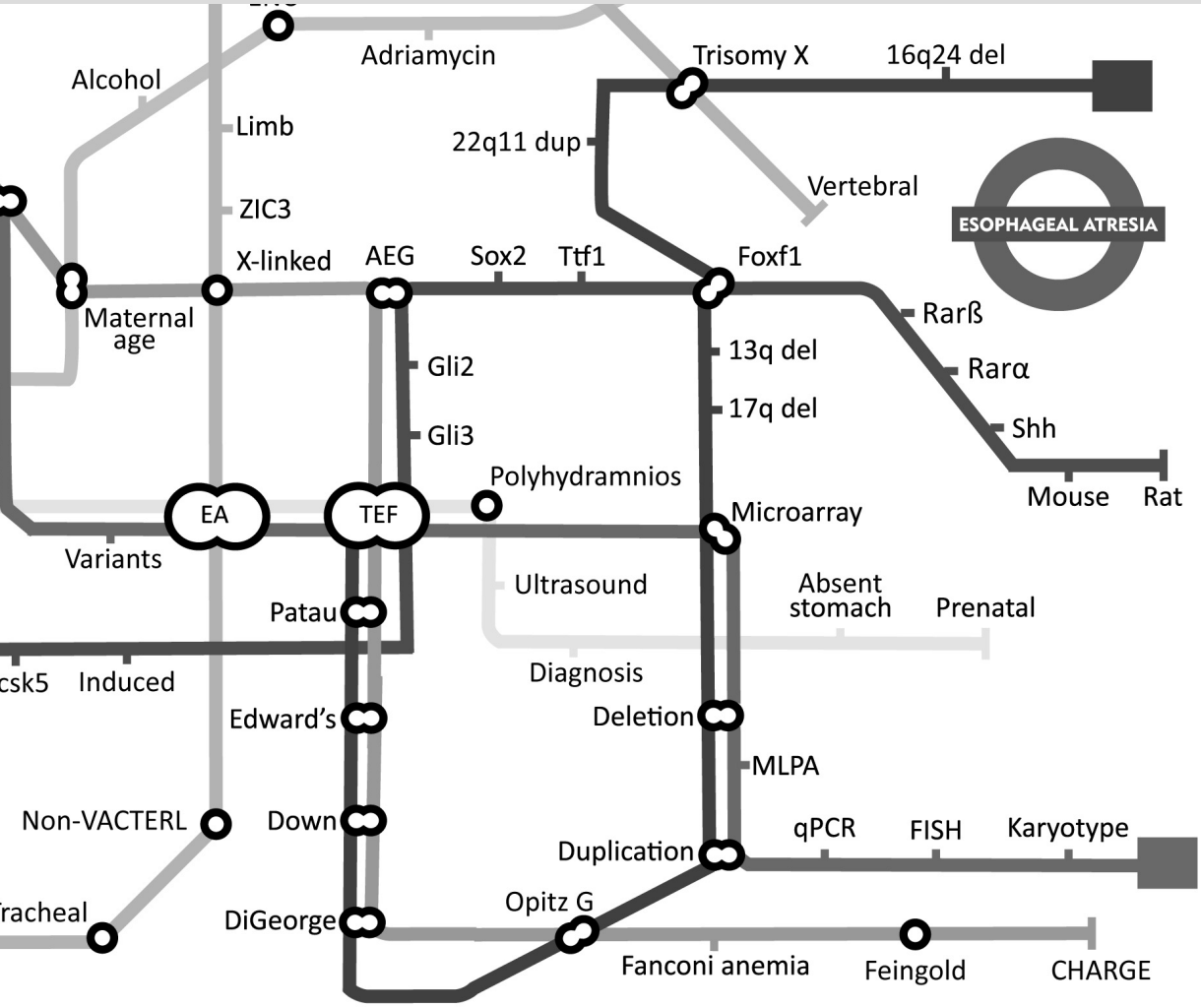
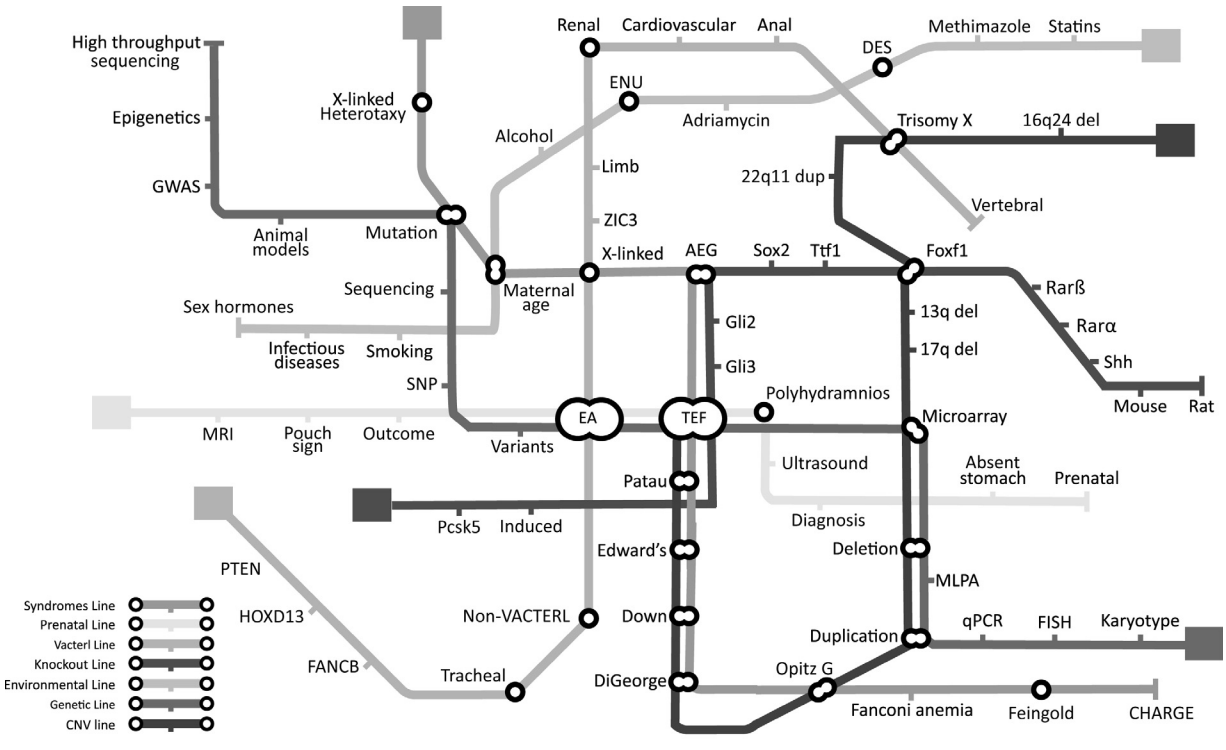
# PART II

## Clinical Studies in Patients with Esophageal Atresia



# CHAPTER 2

## Pre- and Postnatal Diagnosis and Outcome of Fetuses and Neonates with Esophageal Atresia and Tracheo-esophageal Fistula




**Adapted from:**

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## Abstract

**Background:** Clinical symptoms and ultrasound signs during pregnancy could suggest the presence of **e**sophageal **a**tresia (EA). However, most often EA is diagnosed postnatally. The aim of our study is to evaluate the course and outcome for prenatally and postnatally diagnosed EA. In addition, we studied the outcome of isolated versus non-isolated EA.

**Methods:** In a retrospective data analysis, ultrasound characteristics, maternal and neonatal variables as well as clinical outcome were compared for fetuses/neonates with prenatal (n = 30) or postnatal (n = 49) diagnosis of EA. Clinical outcome in terms of morbidity and mortality of isolated EA was compared with that of EA complicated by chromosomal or structural anomalies.

**Results:** Prenatally diagnosed children were born two weeks earlier than postnatally diagnosed children (36.4 weeks versus 38.2 weeks; P = 0.02). The former had higher mortality rates (30% versus 12%; P = 0.05) and more associated anomalies (80% versus 59%; P = 0.04). In both subsets, there was a high morbidity rate in the survivors (not significant). Non-isolated EA was associated with greater occurrence of polyhydramnios (53% versus 27%; P = 0.04) and higher mortality rate (28% versus 0%; P = 0.002).

**Conclusions:** Mortality was significantly higher in prenatally diagnosed infants and in infants with additional congenital anomalies. Isolated EA is associated with good outcome.

## Introduction

Improvements in ultrasound imaging techniques have enabled detection of a range of developmental disorders and congenital malformations early in pregnancy[1]. One such malformation is **e**sophageal **a**tresia (EA), a condition in which the esophagus is interrupted and may or may not communicate with the trachea, through a fistulous tract called the **t**racheo-**e**sophageal **f**istula (TEF). The incidence of EA is approximately one in 3,500 live births and EA with distal TEF is the commonest type (86%)[2-4]. The etiology of tracheo-esophageal anomalies is largely unknown, but is considered to be multifactorial, comprising genetic and environmental susceptibility factors, as reviewed recently[5,6]. Indeed, from 6 to 10% of postnatally diagnosed infants with EA are also diagnosed with a chromosomal abnormality or syndrome[7]. Over 50% of infants with EA have additional anomalies, e.g. vertebral, gastro-intestinal, cardiovascular, renal or limb abnormalities[4]. In approximately 10% of cases, these associated anomalies fit the VACTERL association, which is defined as EA associated with at least two anomalies belonging to the following subsets: vertebrae, anal region, cardiovascular system, renal system or limbs[7,8].

Prenatal detection of EA is difficult. The presence of a polyhydramnios may suggest gastro-intestinal obstruction, but is also related to a wide variation of other anomalies[9,10]. Polyhydramnios combined with a small or invisible stomach raises the positive predictive value to 39-56%[10-14]. This combination lacks specificity, however, because amniotic fluid may flow into the stomach when a TEF is present. Efforts to improve the detection rate have focused on ultrasound or MRI demonstration of an esophageal pouch, i.e. a dilatation of the blind-ending upper esophageal segment[10,15-19]. A new ultrasonographic technique makes it possible to visualize the fetal thoracic esophagus between the trachea and the aorta[20].

Postnatally, EA is suspected in the presence of excessive oropharyngeal secretions, with or without respiratory difficulties, and the inability to pass a nasogastric tube, confirmed on chest radiograph. The two ends of the esophagus are usually surgically anastomosed within 24-48 hours from the diagnosis. Post-operative complications usually include anastomotic strictures and leaks, recurrent tracheo-esophageal fistula, gastro-esophageal reflux and sequelae of thoracotomy, such as scoliosis and thorax asymmetry[4,21,22].

Better diagnostic techniques and pre- and post-operative care have improved the prognosis for these infants[4]. Mortality rates are related to additional congenital malformations and prematurity[4,23,24]. Morbidity is largely the result of postoperative complications, structural abnormalities of the airways and the presence of additional congenital anomalies[4,7,22,25].

Little has been published about the value of a prenatal diagnosis of EA with regard to outcome. We report a study with a twofold aim: (1) to investigate the disease course and outcome of fetuses and neonates with either a prenatal or postnatal diagnosis of EA; and (2) to analyze the influence of additional anomalies on outcome.

## Methods

The Erasmus University Medical Center (Erasmus MC) serves as the single referral center for both fetal anomaly scanning and pediatric surgery in the south-west of The Netherlands, a referral area with 44,000 newborns annually. EA fetuses and infants were identified from the ultrasound (US) databases of the division of Prenatal Medicine and the departments of Pediatric Surgery and Pathology in Erasmus MC as well as from those of referring hospitals. A retrospective data analysis was carried out concerning all fetuses/ infants born between January 2000 and December 2006.

EA in cases of termination of pregnancy (< 24 weeks), fetal or neonatal death, had been diagnosed, only if parents granted permission for post-mortem examination. Post-mortem examinations had not been done in pregnancies complicated by a chromosomal anomaly (e.g. trisomy 18). Infants with isolated fistula were excluded. All infants had received standardized care, which included further examination for congenital anomalies, surgery, and genetic consultation with karyotyping.

Polyhydramnios was defined as an amniotic fluid index >24 cm, measured in four quadrants of the uterus[26]. Absent or small stomach was diagnosed with repeated US examinations. Prematurity was defined as delivery before 37 weeks' gestation. Small for gestational age was defined as < -2SD after correction for gestational age and gender[27]. Isolated EA was defined as EA without additional congenital anomalies and the patient having a normal karyotype. VACTERL association was defined as two or more anomalies of the spectrum, besides the tracheo-esophageal malformation[8].

Two subsets were created: prenatal and postnatal diagnosis. The prenatal subset consisted of all women with documented polyhydramnios, and/or absent or small stomach bubble on ultrasound, detected in Erasmus MC, or in the referring hospitals with confirmed EA. No 3D-ultrasound or MRI investigation was performed. The postnatal subset consisted of all infants admitted to the department of Pediatric Surgery with an EA, without prenatal US examination or without any documentation of the defined complaints during pregnancy. These subsets were compared with respect to outcome and associated anomalies. The outcome

measures were neonatal morbidity (anastomotic esophageal strictures and leaks, recurrent TEF, gastro-esophageal reflux, number of re-operations, length of hospital stay) and mortality. Follow up analysis covered the first 12 months after birth.

In addition, we created subsets of cases with isolated EA and cases with EA and associated anomalies (non-isolated EA). These were likewise compared with respect to morbidity and mortality.

Statistical analysis was performed with SPSS version 15.0.0. using the Mann-Whitney test or Fisher's exact test for continuous or categorical data, respectively. $P < 0.05$ (two-sided) was considered the limit of statistical significance.

## Results

### *Post mortem cases*

Three pregnancies had been terminated (< 24 weeks) after prenatal US had revealed multiple severe structural anomalies. One was terminated at 21 3/7 weeks and concerned a male fetus with an EA with distal fistula, associated with duodenal atresia and an eventration of the diaphragm. Karyotype was normal and no dysmorphic features were seen. The second pregnancy, terminated at 22 3/7 weeks, was complicated with oligohydramnios. The male fetus had an EA with distal fistula, associated with anal atresia, agenesis of the left kidney, a dysplastic right kidney, pre-axial polydactyly, bilateral pes equinovarus and dysmorphic features, e.g. low implant of ears with malformed ear helixes and a single umbilical artery. The third pregnancy had been terminated at 19 1/7 weeks. Multiple structural abnormalities and intrauterine growth retardation were seen on US. The female fetus had EA with distal fistula, associated with a hypoplastic left heart, mono-ventricle with common outlet, uterus bicornis and dysmorphic features; agenesis of the left ear and dysplastic right ear, prognathy, small mandible and an asymmetric face. Ultrasound characteristics of EA and/or polyhydramnios were seen in none of these three cases, most likely due to the gestational age at termination.

### *Characteristics of the prenatal subset*

In total, the prenatal subset consisted of 17 fetuses diagnosed at Erasmus MC and 13 fetuses from referring hospitals. Ultrasound variables of fetuses diagnosed at Erasmus MC were measured in a standardized manner, and data were stored in a prenatal database (Table 1). Three of these 17 infants had an isolated EA (18%). The other 14 showed one or more additional anomalies, mainly vertebral, cardiac, renal, or anorectal anomalies. The US diagnosis of EA had

been made significantly earlier in the cases of isolated EA (26.4 versus 32 weeks; P = 0.04). AFI was > 30 in 8 of these 17 infants (47%). There was no significant difference in this regards between infants with isolated EA and non-isolated EA. One chromosomal anomaly was found (trisomy 21). The prognosis was good for patients with isolated EA; none of these infants died in the postnatal period, versus 6 of the 14 in the non-isolated group (43%). The presence of associated anomalies had no effect on postnatal morbidity with regard to duration of hospitalization, number of complications and number of re-surgeries.

From the information provided by obstetricians from the referring hospitals about the remaining 13 infants, it appeared that all women showed symptoms of polyhydramnios. In three patients the stomach bubble was absent. However, more detailed ultrasound descriptions or data were lacking.

**Table 1: Characteristics of the prenatal subset of patients with EA from Erasmus MC**

| | **Isolated EA N=3** | **Non-isolated EA N=14** | **P value** |
|---|---|---|---|
| GA ultrasound weeks, mean (range) | 26.4 (20.0-32.3) | 32.0 (25.4-36.1) | 0.04* |
| GA ultrasound < 30 weeks (%) | 2 (67) | 5 (36) | 0.57 |
| AFI, mean† | 28.4 | 31.5 | 0.39 |
| AFI > 30 (%) | 1 (33) | 7 (50) | 1.00 |
| Spontaneous delivery (%) | 2 (67) | 8 (56) | 1.0 |
| GA delivery weeks, mean (range) | 38.0 (32.3-41.1) | 35.3 (29.1-42.0) | 0.31 |
| Birth weight g, mean (range) | 2335 (1640-2825) | 2328 (1060-3440) | 1.0 |
| Neonatal death (%) | 0 | 6 (43) | 0.52 |
| Male gender (%) | 2 (67) | 9 (64) | 1.0 |
| Anomalies (%) | - | | |
| – Vertebral | | 2 (14) | |
| – Anorectal | | 4 (28) | |
| – Cardiac | | 6 (42) | |
| – Renal | | 5 (36) | |
| – Limb | | 1 (7) | |
| Chromosomal anomaly | - | 1 | |
| Length of hospital stay, mean (range)$^{\$}$ | 54.7 (19-86) | 114.3 (28-325) | 0.22 |
| No. of complications | 0.7 | 0.9 | 0.33 |
| No. of surgeries | 4.3 | 3.7 | 0.62 |

GA, gestational age; †, excluded anhydramnios due to solutio placenta or PROM; *, statistically significant; $, excluded neonatal death

*Comparison of prenatal and postnatal subsets of EA patients*

The postnatal subset encompassed 49 patients, without prenatal US examination or without any documentation of complaints during pregnancy. Delivery and outcome variables for both subsets are shown in Table 2. The mean gestational age at delivery in the prenatal subset was significantly shorter (36.4 weeks) than that in the postnatal subset (38.2 weeks; P = 0.02). This was not due to induction of labour; the mode of delivery did not differ between the two subsets. Consequently, infants from the prenatal subset had a significantly (P = 0.03) lower mean birth weight (2459 g) than the infants from the postnatal subset (2873 g). The proportion of infants with an Apgar Score ≤ 6 at 5 minutes in the prenatal subset (5/30) was significantly higher than that in the postnatal subset (0/49) (P = 0.006). The proportion of infants with an isolated EA was highest in the postnatal subset (6/30 versus 20/49; P = 0.04). A high percentage of associated anomalies was seen in both subsets; VACTERL association was seen in 30% of the prenatal subset and in 27% of the postnatal subset. The prenatal subset had a higher proportion of infants with a genetic syndrome (5/30 versus 1/49; P = 0.03).

The mortality in the prenatal subset was highest (9/30 versus 6/49; P = 0.05). Three of the nine deceased patients in the prenatal subset had CHARGE syndrome (**C**oloboma of the eye, **H**eart defects, **A**tresia of the choanae, **R**etardation of growth and/or development, **G**enital and/or urinary abnormalities, and **E**ar abnormalities and deafness), one patient had Down syndrome and the other five died from severe associated congenital anomalies, including cardiovascular, lung and renal defects.

Post-operative complications in the survivors were frequent in both subsets, e.g. 14/21 of infants from the prenatal subset (67%) and 25/43 from the postnatal subset (58%) had gastro-esophageal reflux. Nine out of 21 infants from the prenatal subset (43%) and 19 out of 43 from the postnatal subset (44%) had esophageal strictures. The two subsets did not differ in number of surgeries, post-operative complications and duration of hospitalization.

*Comparison of patients with isolated and non-isolated EA* (Table 3)

Twenty-six of the 79 infants (33%) presented with isolated EA; 53 (67%) presented with EA and additional anomalies. A significantly larger proportion of patients in the non-isolated subset showed polyhydramnios (7/26 versus 28/53; P = 0.04), independent of the presence of a tracheo-esophageal fistula. The gestational age at delivery was significantly lower in the non-isolated subset (38.2 weeks versus 37.2 weeks; P = 0.01). There were no significant differences in the prevalence of prematurity (6/26 versus 17/53) or induced labour (7/26 versus 19/53). Neither did birth weight and Apgar scores differ significantly. The prevalence of VACTERL association is high (22/53; 42%).

**Table 2: Outcome data of the prenatal and postnatal subset with esophageal atresia**

| | Prenatal subset N = 30 | Postnatal subset N = 49 | P- value |
|---|---|---|---|
| GA at delivery weeks, mean (range)[†] | 36.4 (29.1-42.2) | 38.2 (28.4-42.1) | 0.02* |
| Non-spontaneous delivery (%)[‡] | 12 (40) | 14 (29) | 0.29 |
| Birth weight, g, mean, (range)[†] | 2459 (1060-3800) | 2873 (1130-4000) | 0.03* |
| SGA (%) | 12 (40) | 11 (22) | 0.10 |
| Male gender (%) | 18 (60) | 30 (61) | 0.91 |
| Apgar 5 ≤ 6 (%) | 5 (16) | 0 | 0.006* |
| EA with tracheo-esophageal fistula (%) | 25 (83) | 45 (92) | 0.29 |
| Isolated esophageal atresia (%) | 6 (20) | 20 (41) | 0.04* |
| Associated anomalies (%) | | | |
| – Vertebral | 5 (16) | 15 (31) | 1.00 |
| – Anorectal | 4 (13) | 7 (14) | 1.00 |
| – Cardiac | 9 (30) | 11 (23) | 0.60 |
| – Renal | 1 (3) | 8 (16) | 0.47 |
| – Limb | 10 (33) | 8 (16) | 0.20 |
| – VACTERL[¶] | 9 (30) | 13 (27) | 0.74 |
| – Non-VACTERL | 12 (40) | 15 (31) | 0.59 |
| – Genetic syndrome[§] | 5 (17) | 1 (2) | 0.03* |
| Neonatal death (%) | 9 (30) | 6 (12) | 0.05* |
| Primary end-to-end anastomosis (%) | 24/26 (92) | 42/45 (93) | 1.00 |
| No. of surgeries | 3.3 | 3.4 | 0.90 |
| Length of hospital stay, mean (range)[$] | 71 (14-325) | 70 (11-365) | 0.65 |
| Complications (%)[$] | 13/21 (62) | 32/43 (74) | 0.39 |
| – Anastomotic leaks | 1 (5) | 3 (7) | 1.00 |
| – Strictures | 9 (43) | 19 (44) | 1.00 |
| – Recurrent fistula | 0 | 3 (7) | 0.55 |
| – Gastro-esophageal reflux | 14 (67) | 25 (58) | 1.00 |
| – Other complications | 4 (19) | 13 (30) | 0.39 |

GA, gestational age; SGA, small for gestational age; [†], Excluding cases of intrauterine fetal death or termination of pregnancy; [‡], Induced delivery and caesarean section; [¶], Besides EA, two or more anomalies of VACTERL spectrum; [§], Down syndrome, CHARGE syndrome and 22q11 deletion syndrome; [$], excluded neonatal death; *, statistically significant

Fifteen patients in the non-isolated subset had died in the neonatal period, versus none in the isolated subset (P = 0.002). Seven of the deceased patients had severe other congenital anomalies, three had been diagnosed with CHARGE syndrome, two died during surgical repair of severe heart defects, two died of sepsis and one died during birth.

Post-operative complications in the survivors were frequent in both subsets, e.g. 16/26 of infants from the isolated subset (62%) and 21/38 infants from the non-isolated subset (55%)

suffered from gastro-esophageal reflux. Esophageal strictures were present in 42% (11/26) and in 45% (17/38) of infants from the isolated and non-isolated subset, respectively. The two subsets did not differ in number of surgeries, post-operative complications and duration of hospitalization.

**Table 3: A comparison of patients with isolated and non-isolated EA**

| | Isolated EA N=26 | Non-isolated EA N=53 | P-value |
|---|---|---|---|
| Polyhydramnios (%) | 7 (27) | 28 (53) | 0.04* |
| GA at delivery, weeks, mean (range) | 38.2 (29.4-40.1) | 37.2 (28.4-42.2) | 0.01* |
| Premature delivery < 37 wk (%)[‡] | 6 (23) | 17 (32) | 0.44 |
| Non-spontaneous delivery (%) | 7 (27) | 19 (36) | 0.50 |
| Birth weight, G, mean (range) | 2846 (1280-4000) | 2652 (1060-4000) | 0.24 |
| Small-for-gestational-age (%) | 7 (27) | 19 (36) | 0.80 |
| Male gender (%) | 18 (69) | 30 (57) | 0.33 |
| Apgar 1 (≤ 6) % | 4 (15) | 10 (19) | 0.76 |
| Apgar 5 (≤ 6) % | 1 (4) | 4 (8) | 0.66 |
| Neonatal death (%) | 0 | 15 (28) | 0.002* |
| EA with tracheo-esophageal fistula (%) | 24 (92) | 46 (87) | 0.71 |
| VACTERL (%)[¶] | - | 22 (42) | |
| Primary end-to-end anastomosis (%) | 24 (92) | 42 (79) | 1.00 |
| No. of surgeries | 2.7 | 3.7 | 0.55 |
| Length of hospital stay, mean (range)[$] | 51 (11-206) | 84 (13-365) | 0.07 |
| Complications (%)[$] | 18/26 (69) | 27/38 (71) | 1.00 |
| – Anastomotic leaks | 2 (8) | 2 (5) | 1.00 |
| – Strictures | 11 (42) | 17 (45) | 1.00 |
| – Recurrent fistula | 2 (8) | 1 (3) | 0.56 |
| – Gastro-esophageal reflux | 16 (62) | 21 (55) | 0.80 |
| – Other complications | 9 (35) | 8 (21) | 0.26 |

[‡], Induced delivery and caesarean section, [¶] Besides EA, two or more anomalies of VACTERL spectrum; [$], excluded neonatal death; *, statistically significant

## Discussion

The present study compared prenatally and postnatally diagnosed infants and their clinical course up to one year of age. Non-isolated EA patients are more likely to be diagnosed prenatally than isolated patients. Overall, the prenatally diagnosed infants were born two

weeks earlier at a significantly lower birth weight, after correction for gestational age. The lower gestational age is likely due to the anomaly combined with polyhydramnios as the mode of delivery did not differ between the two subsets. It would seem, therefore, that pregnancies in the prenatally diagnosed subset were more complicated.

The mortality rate for the prenatally diagnosed infants was significantly higher than for postnatally diagnosed infants. The former also seem to be more severely affected, as this subset included a significantly higher percentage of infants with additional anomalies. However, an expected higher, EA-related morbidity rate for infants with additional anomalies could not be established. These findings do support our clinical experience that infants with isolated EA have a better prognosis, as also suggested in the literature[11,23,28].

For only one infant in the prenatal subset a chromosomal anomaly had been documented, i.e. trisomy 21. However, in all pregnancies terminated before 24 weeks of gestation because of a chromosomal anomaly, no standard autopsy was done. As the prenatal symptoms of an EA occur mainly in the third trimester of pregnancy, the prevalence of EA may be underestimated.

VACTERL associated anomalies were seen in over 40% of patients with non-isolated EA, and thus in 28% of the total group. The prenatal and postnatal subsets did not differ in this regard (P = 0.74). Consistent with other studies the prenatal subset included a higher number of patients with syndromic EA[12,14,29,30].

All infants received standardized care and were followed for at least one year. While mortality differed significantly (P = 0.002) there were neither differences in complications nor in EA related morbidity between survivors with isolated EA and those with complicated EA. In contrast to the recent study by Brantberg *et al.*, we found a significantly higher mortality in infants in whom EA was suspected prenatally[9]. As a large proportion of the postnatally diagnosed infants did not receive tertiary antenatal care, it may be doubted whether our prenatal findings truly reflect the prediction rates of our center. In addition, we saw significant differences for the presence of polyhydramnios and for gestational age of delivery in the non-isolated subset. We would recommend improving tertiary care for mothers with complicated pregnancies, i.e. adequate referral and good postnatal care, as a means to improve survival.

In conclusion, the prognosis of isolated EA is good. Morbidity in all survivors is high, however, independent of time of diagnosis and of the presence of associated anomalies. The mortality rate for infants diagnosed prenatally is significantly higher than for infants diagnosed postnatally. Parental counseling as to the likelihood of EA should be undertaken cautiously and with the input of a multi-disciplinary team, thus allowing for informed choice of center of birth and postnatal care.

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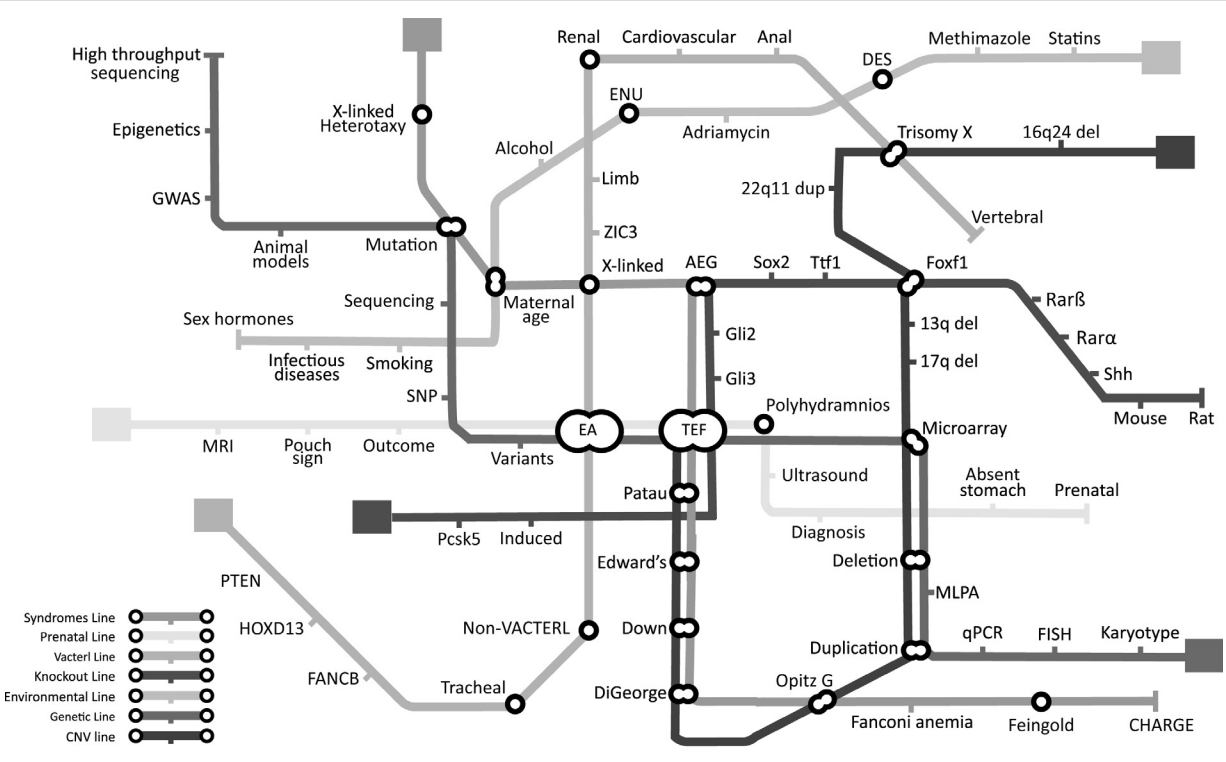
# CHAPTER 3

## Non-VACTERL-type Anomalies are Frequent in Patients with Esophageal Atresia/ Tracheo-esophageal Fistula and Full or Partial VACTERL Association




**Adapted from:**

**de Jong EM,** Felix JF, Deurloo JA, van Dooren MF, Aronson DC, Torfs CP, Heij HA ,Tibboel D. 2008. Non-VACTERL-type anomalies are frequent in patients with esophageal atresia/tracheo-esophageal fistula and full or partial VACTERL association. *Birth Defects Res A Clin Mol Teratol* 82:92-97.

## Abstract

**Background:** The VACTERL association is the non-random co-occurrence of **V**ertebral anomalies, **A**nal atresia, **C**ardiovascular malformations, **T**racheo-**e**sophageal **f**istula (TEF) and/or **E**sophageal **a**tresia (EA), **R**enal anomalies and/or **L**imb-anomalies. The full phenotype of patients with EA/TEF and other anomalies of the VACTERL spectrum of defects association is not well described in the literature.

**Methods:** Data on patients with EA/TEF seen in two pediatric surgical centers in the Netherlands between January 1988 and August 2006 were evaluated for defects of the VACTERL spectrum as well as non-VACTERL-type defects. The presence of two or more defects of the VACTERL spectrum in addition to EA/TEF was the criterion for inclusion in this study. A detailed description was made of all defects.

**Results:** Of 463 patients with EA and/or TEF, 107 (23.1%) fulfilled the inclusion criterion, of which seventeen cases had a recognized etiology and were excluded, leaving 90 cases (19.4%) for analysis. Other than the esophagus and the trachea, the vertebrae/ribs and the cardiovascular system were most commonly affected (68.9 and 65.6%, respectively). Interestingly, 70% of cases had additional non-VACTERL-type defects, with high occurrences for single umbilical artery (20%), genital defects (23.3%) and respiratory tract anomalies (13.3%).

**Conclusions:** Many patients with EA/TEF and at least two other defects of the VACTERL spectrum also display non-VACTERL-type congenital anomalies.

## Introduction

Quan and Smith in 1973 introduced the acronym VATER for a sporadic constellation of birth defects that occur together in the same infant more often than by chance alone[1]. VATER stands for **V**ertebral defects, **A**nal atresia, **T**racheo-**E**sophageal **f**istula (TEF) and/or **e**sophageal **a**tresia (EA), **R**enal anomalies and **R**adial dysplasia. Later, **c**ardiac (C) and **l**imb (L) defects were added and the acronym was extended to VACTERL[2]. The inclusion of cardiac defects is still debated, however, as is the number of defects needed for the diagnosis of a VACTERL association[3-9]. Reported prevalences of a full or partial VACTERL association range from 0.3 to 2.1 per 10,000 live births, depending on the clinical definition used and the population studied[3,10,11].



Other defects, such as cleft lip and/or palate and urogenital anomalies occur more frequently than expected in conjunction with the VACTERL association, but are usually not considered to be part of it[4,9,10,12].

The etiology of the VACTERL association is still unclear and is considered to be multifactorial. A few specific chromosomal anomalies, such as distal 13q deletions and 17q deletions, have been described in patients displaying characteristics of the VACTERL spectrum, but no single chromosomal defect has been shown to play an etiologic role[13-16].

*Sonic hedgehog (shh)* knockout mice show defects comparable to the VACTERL phenotype. However, anomalies of *SHH* have not specifically been associated with the VACTERL association in humans[17].

Environmental exposures during pregnancy, such as exogenous sex hormones during the first trimester of pregnancy, have been suggested as risk factors[2]; however no single environmental risk factor has consistently been identified[8,18-20]. When Adriamycin, an antibiotic used in chemotherapy, is given to pregnant rats, it produces many anomalies of the VACTERL-type in the offspring[21]. So far, however, the association of Adriamycin or structurally related substances with VACTERL-type anomalies has not been observed in humans[22-24].

The reported proportions of patients with the full or partial VACTERL spectrum of defects that includes EA/TEF range from 24 to 67%[4,9,10,12]. However, only five papers describe this specific group of patients[9,25-28]. Previous studies either describe a small sample of cases[25,26], or do not describe non-VACTERL-type defects[28], or use a wide definition of VACTERL association[27].

In this study, we evaluated a large sample of patients with EA/TEF for the occurrence of defects of the VACTERL spectrum and of additional non-VACTERL-type structural malformations in particular. Our rationale was to give a detailed description of all anomalies present in these patients, who may share a common, but so far unknown, etiology[29].

## Methods

We evaluated data stored in the databases of the Erasmus MC – Sophia Children's Hospital in Rotterdam and the Pediatric Surgical Center of Amsterdam (Emma Children's Hospital AMC and VU University Medical Center), both located in The Netherlands. The pediatric surgical departments of these university hospitals are among the six tertiary referral centers for pediatric surgery in The Netherlands. All children born with EA/TEF in The Netherlands are treated in one of these centers. The Sophia Children's Hospital is the pediatric referral center for the Southwestern part of The Netherlands and has the only specialized pediatric surgical intensive care unit in The Netherlands. The Pediatric Surgical Center of Amsterdam is the referral center for pediatric surgical patients in the Northwestern provinces. Together, these hospitals cover almost half of the population of The Netherlands and around 70,000 births annually. All infants with EA/TEF born in the referral areas are admitted to either of the two hospitals. Both centers keep comprehensive databases on cases with EA/TEF, including data on associated anomalies, pregnancy history, and postnatal clinical course.

As a criterion for the diagnosis of the VACTERL association, we used the presence of EA/TEF and at least two additional defects of the VACTERL-spectrum, namely, vertebral anomalies (including rib anomalies), anal atresia, cardiovascular malformations, renal anomalies or radial type limb anomalies.

Karyotyping by G-banding with a resolution of at least 550 bands was performed in patients on clinical grounds. Treating physicians decided if this was done. If a patient died, an autopsy was performed if the parents consented.

After approval from the medical ethics review boards of both hospitals, the databases were searched for patients born from January 1988 up to and including August 2006, who fulfilled these criteria. Cases with known syndromes and chromosomal anomalies were excluded from the analysis.

Medical records from all patients whose defects fulfilled the criteria were checked for the presence of associated non-VACTERL-type anomalies. Undescended testis was not classified as a congenital anomaly in infants who were born preterm. Data were analyzed using SPSS 12.0.1.

## Results

A total of 463 patients with EA and/or TEF were admitted to either of the two centers from January 1988 to August 2006. Of these, 107 (23.1%) patients had two or more additional VACTERL-type defects. Seventeen patients with a recognized etiology, such as a syndrome or a chromosomal disorder, were excluded (Table 1). Of the 17 excluded patients, 13 had chromosomal aberrations, one had a gene mutation and three had a syndrome without (known) chromosomal or genetic aberrations. This left 90 (19.4%) cases for analysis, which included 58 (64.4%) males and 32 (35.6%) females. Using the Gross classification, we identified four (4.4%) cases with type A (isolated EA), one case (1.1%) with type B (EA with proximal TEF), 81 (90.0%) with type C (EA with distal TEF), two cases (2.2%) with type D (EA with proximal and distal TEF) and one (1.1%) case with TEF without EA (type E)[30]. One patient with type C had two distal fistulas. The type was not recorded in one patient.



**Table 1: Chromosomal anomalies & syndromes found in 107 cases with EA/TEF and at least 2 additional defects of the VACTERL spectrum of anomalies**

| Chromosomal anomaly / syndrome | No. of cases |
|---|---|
| Down syndrome (trisomy 21) | 5 |
| Edwards syndrome (trisomy 18) | 3 |
| Patau syndrome (trisomy 13) | 2 |
| Goldenhar syndrome | 1 |
| 22q11 deletion | 1 |
| Inversion chromosome 9 | 1 |
| Smith Lemli Opitz syndrome | 1 |
| Townes Brocks syndrome | 1 |
| 46,XY,t(5;10)(q13;q23)pat | 1 |
| Tbx5 mutation | 1 |
| Total | 17 |

Gestational ages ranged from 26 4/7 to 42 3/7 weeks (median 37 4/7 weeks). Thirty-five cases (38.9%) were born preterm (< 37 weeks) and six cases (6.7%) were post term (42 weeks or more). Birth weights ranged from 775 to 3825 g (mean 2420 g). Twenty-five cases (27.8%) were small for gestational age (more than two standard deviations below the mean). Gestational age and birth weight data were missing for one patient.

A total of 25 patients (27.8%) died, of whom 13 (52.0%) at a neonatal age (median age of death 4 days, range 0-21 days) and 11 (44.0%) at an infant age (median age of death 142 days, range 40-1971 days). For one child, there was no information on age at death.

Exact causes of death are not recorded in the databases. One of the 25 cases had all six anomalies of the VACTERL spectrum (full VACTERL association), three had five anomalies, five had four anomalies and 16 had three. Twenty-two cases had a cardiac defect, of whom six had more than one cardiac defect. Four patients had severe anomalies of the respiratory tract, including one case with tracheal agenesis, and three had central nervous system anomalies, one of them a myelomeningocele. Of course, these anomalies are not mutually exclusive and the combination of defects has likely contributed to the death of those children, taking into account withdrawal of treatment in selected cases. Autopsy was performed on 10 (40%) of the patients who died. This did not reveal new congenital anomalies.

Karyotyping was performed for 67 (74.4%) cases. As children with chromosomal anomalies were excluded from the analysis (see Table 1), all 67 children that were examined had a normal karyotype. One of our patients had an older brother with isolated EA/TEF.

Table 2 gives an overview of the VACTERL-type defects other than EA/TEF, found in the 90 studied cases. The vertebrae/ribs and the cardiovascular system were most commonly affected (68.9 and 65.6%, respectively). Of the 90 cases, 59 (65.6%) had three defects, 22 (24.4%) had four defects, seven (7.8%) had five defects and two (2.2%) patients (both girls) were diagnosed with all six VACTERL components.

Interestingly, while only 27 cases (30.0%) had only VACTERL-type defects, as many as 63 cases (70.0%) had VACTERL-type defects in association with other structural defects (Table 3). A high occurrence of a single umbilical artery (20.0%) and of genital defects (23.3%) was observed. There was only one preterm infant with undescended testis and without other genital anomalies, so this child was not included in the 'genital' group. Other associated defects included respiratory tract anomalies (13.3%), duodenal atresia (8.9%) and cleft lip/jaw/palate (4.4%).

**Table 2: Defects of the VACTERL spectrum of anomalies found in 90 cases with EA/TEF**

| Defect | No. of cases (%) |
|---|---|
| **Vertebral** | **62 (68.9)** |
| – Vertebral anomalies | 50 (55.6) |
| – Rib anomalies | 37 (41.1) |
| **Anal** | **38 (42.2)** |
| – Anal atresia/stenosis/web | 31 (34.4) |
| – Abnormal placement of anus | 7 (7.8) |
| **Cardiovascular** | **59 (65.6)** |
| – Atrial septum defect | 32 (35.6) |
| – Ventricular septum defect | 23 (25.6) |
| – Atrio-ventricular septal defect | 4 (4.4) |
| – Fallot tetralogy | 9 (10.0) |
| – Double outlet right ventricle | 1 (1.1) |
| – Hypoplastic left ventricle | 2 (2.2) |
| – Hypoplastic right ventricle | 1 (1.1) |
| – Univentricular heart | 1 (1.1) |
| – Anomalies of the cardiac valves | 3 (3.3) |
| – Transposition of great arteries | 1 (1.1) |
| – Coarctation/interrupted aorta | 3 (3.3) |
| – Double aortic arch | 1 (1.1) |
| – Other aortic arch defects | 2 (2.2) |
| – Dextrocardia | 3 (3.3) |
| – Arteria lusoria | 2 (2.2) |
| **Renal** | **32 (35.6)** |
| – Hydronephrosis | 7 (7.8) |
| – Horseshoe kidneys | 7 (7.8) |
| – Renal agenesis | 8 (8.9) |
| – Renal cysts | 6 (6.7) |
| – Small kidneys | 3 (3.3) |
| – Renal dysplasia | 3 (3.3) |
| – Division of renal pelvis | 1 (1.1) |
| – Ectopic kidneys | 3 (3.3) |
| – Ureteral anomalies | 2 (2.2) |
| – Pyelo-ureteral junction stenosis | 3 (3.3) |
| **Upper Limb** | **29 (32.2)** |
| – Radial anomalies | 16 (17.8) |
| – Thumb anomalies | 18 (20.0) |
| – Preaxial polydactyly | 6 (6.7) |

Defects described are not mutually exclusive

**Table 3: Associated non-VACTERL-type structural anomalies found in 90 cases with EA/TEF**

| Anomaly | Number of cases (%) |
|---|---|
| **Single umbilical artery** | **18 (20.0)** |
| **Duodenal atresia/web** | **8 (8.9)** |
| **Cleft lip/jaw/palate** | **4 (4.4)** |
| **Genital anomalies** | **21 (23.3)** |
| – Testicular anomalies, including undescended testis | 7(7.8) |
| – Penile anomalies, including hypospadias | 9 (10.0) |
| – Bifid scrotum | 3 (3.3) |
| – Uterine anomalies | 2 (2.2) |
| – Anomalies of clitoris | 3 (3.3) |
| – Absent ovaries | 1 (1.1) |
| – Double vagina | 1 (1.1) |
| – Hydrocolpos | 2 (2.2) |
| – Abnormal labia | 1 (1.1) |
| – Ambiguous genitalia | 2 (2.2) |
| – Cloacal malformation | 2 (2.2) |
| **Urinary tract anomalies** | **14 (15.6)** |
| – Vesico-urinary reflux | 11 (12.2) |
| – Patent urachus | 1 (1.1) |
| – Bladder anomalies | 3 (3.3) |
| – Urethral anomalies, including valves | 3 (3.3) |
| **Respiratory system anomalies** | **12 (13.3)** |
| – Laryngeal/tracheal/bronchial anomalies | 7 (7.8) |
| – Lung hypoplasia/agenesis | 2 (2.2) |
| – Cricoid stenosis | 1 (1.1) |
| – Choanal atresia | 1 (1.1) |
| – Uvular anomalies | 2 (2.2) |
| **Intestinal anomalies** | **8 (8.9)** |
| – Intestinal malrotation | 3 (3.3) |
| – Small bowel atresia/hypoplasia | 1 (1.1) |
| – Meckel's diverticulum | 4 (4.4) |
| **Lower limb anomalies** | **15 (16.7)** |
| – Hypotrophy lower limbs | 1 (1.1) |
| – Dysplastic hip(s) | 2 (2.2) |
| – Club foot | 4 (4.4) |
| – Rocker-bottom feet | 1 (1.1) |
| – Anomalies of toes | 10 (11.1) |

| Anomaly | Number of cases (%) |
|---|---|
| **Skeletal (other)** | **10 (11.1)** |
| – Upper limbs, non-VACTERL defects | 9 (10.0) |
| – Abnormally shaped thorax | 1 (1.1) |
| – Skull anomalies | 1 (1.1) |
| **Vascular anomalies (other)** | **10 (11.1)** |
| – Abnormal arterial supply right lung | 1 (1.1) |
| – Anomalies of vena cava | 5 (5.6) |
| – Right descending aorta | 3 (3.3) |
| – Anomalous venous return | 2 (2.2) |
| **Nervous system anomalies** | **10 (11.1)** |
| – Anomalies of spinal cord, including spina bifida | 6 (6.7) |
| – Structural brain anomalies | 5 (5.6) |
| **Other anomalies** | **6 (6.7)** |
| – Polysplenia | 2 (2.2) |
| – Pancreatic anomalies | 2 (2.2) |
| – Abnormal position gallbladder | 1 (1.1) |
| – Left isomerism | 1 (1.1) |
| – Peters anomaly | 1 (1.1) |

Anomalies are not mutually exclusive



## Discussion

This study, based on data from Pediatric Surgical Centers, describes a group of 90 patients with both EA/TEF and at least two other defects included in the VACTERL spectrum. The defects are well documented, thanks to the routine screening of children with EA/TEF for VACTERL-type defects by more detailed, mainly non-invasive, diagnostic techniques (e.g., renal and cardiac ultrasound) since the late 1980s. All patients in our study were screened for other VACTERL-type defects by X-rays of the vertebrae and ribs, and by cardiac and renal ultrasound. Further imaging techniques were only done on clinical grounds and were not part of the standard screening.

The most remarkable finding in our population was that as many as 70% of all patients showed defects other than those included in the VACTERL association. The corresponding figures reported by other authors are often much lower, with a maximum of 57%, reported by Czeizel *et al.*[9,25].

In a study of the VACTERL association that included all cases with any three or more of the six defects, but not necessarily EA/TEF, non-VACTERL-type anomalies were described in 20%[10]. The high numbers reported in our population may reflect the high percentage of patients with EA/TEF that have associated anomalies in general (around 50%)[25,31]. In addition, our population of cases is very well documented, as many cases were also assessed by a clinical geneticist/dysmorphologist, thus increasing the likelihood of reporting minor anomalies. However, most observed non-VACTERL-type defects were not minor anomalies that would be missed on routine clinical examination.

Comparing our results to those of others is not an easy task. Several of the defects associated with EA/TEF are also associated in the absence of EA/TEF[3,7,32]. However, we focused on defects associated with EA/TEF and only describe those patients with EA/TEF and at least two other VACTERL-type defects, according to our definition of full or partial VACTERL association. There are only a few papers in the literature reporting data from populations similar to ours[9,25,26,28,33].

In a study that focuses on 1846 infants with anorectal defects, Cuschieri *et al.* found 181 cases who also had one or more of the VACTERL associated type of defects. Fifty seven of these also had EA/TEF, showing the association of these two types of defects from the perspective of anorectal defects[34]. Our study parallels theirs, but starts with a population of infants with EA/TEF instead of anorectal defects.

In our population, 20% of cases had a single umbilical artery (SUA). The corresponding figure by Chittmittrapap *et al.* reported 8%, but Temtamy and Miller, in a smaller group, described an incidence of 70%[35]. In a study based on data for 292 cases of EA/TEF in the United States, Torfs *et al.* reported a strong association between SUA and EA/TEF in general, with SUA found in 18.2% of cases[28]. This population, however, included 36 cases with aneuploidies and syndromes. Nevertheless, none of the 28 trisomy cases had a SUA[28] and exclusion of these cases would therefore bring up this proportion to 20%, which is similar to our finding.

We found duodenal atresia in 8.9% of our population, and cleft lip, jaw and/or palate in 4.4%. One of the two other studies with data comparable to ours reports duodenal atresia in a similar proportion of cases (8.0%), but does not report cases of clefts[25]. The other comparable study, that by Czeizel *et al.*, found cleft lip and/or palate in 13.2% and intestinal atresia (n.o.s.) in 2.6%[9]. In studies of associated anomalies in children with EA/TEF, cleft lip and palate were reported in 2.6% and 2.7% of patients[36,37]. Keckler *et al.* recently described cleft palate in 4.5% and cleft lip in 0.9% of cases. These latter numbers are very similar to ours, yet the population is somewhat different[33].

In our study, anomalies of the respiratory system were present in 13% of cases. This finding is not entirely surprising, as both the esophagus and the respiratory system develop from the foregut. The presence of EA/TEF may be a sign of defective foregut development in general, which could also lead to respiratory tract anomalies. A number of mouse knockout models that show EA/TEF also display anomalies of the respiratory system, e.g. knockout mice for either *sonic hedgehog*, *gli2/gli3* or *Ttf-1*[38-40]. In a group of 25 cases with EA/TEF and at least two other defects of the VACTERL-spectrum, Chittmittrapap *et al.* described the absence of the right upper lobe of the lung in one patient (4%)[25]. Temtamy reported the occurrence of unilateral lung agenesis in an unspecified number of cases among a similar, but smaller, group of 10 cases of EA/TEF combined with at least two other VACTERL-type defects[26]. Czeizel *et al.* found lung hypoplasia/agenesis in 7.9% of their population of patients with EA/TEF and at least two other anomalies of the VACTERL-spectrum[9].



We observed that 19.4% of 463 infants with EA/TEF had at least two additional defects of the VACTERL-spectrum, a proportion that falls in the range of prevalences (10-32%) reported by others[25,28,35,41]. We found 64.4% of all these cases to be boys, which compares with proportions reported earlier[9,25].

Only a small proportion of patients had the whole spectrum of six anomalies, thereby representing the 'full' VACTERL association. Although several studies on the VACTERL association include patients with only two of the anomalies of the VACTERL spectrum[3,12,19], most studies include only patients with three or more of the defects[4,8,9,25,32,35]. We have adopted the latter, more stringent definition with the restriction that EA/TEF always isincluded.

Therefore, most patients in our study have a 'partial' VACTERL association. Our data do not permit to draw conclusions about the number of defects, nor which defects of the spectrum must necessarily be included for the term 'VACTERL' to apply. Also, some studies exclude patients who fulfill the criteria for VACTERL association, but who have been diagnosed with a specific chromosomal anomaly or syndrome[10,12]. The same decision was made in the present study. However, others have included such cases, which makes comparison between studies more difficult[27].

In many centers, karyotyping is now standard practice for children with multiple congenital anomalies or even for all children with one or more major congenital anomalies. In addition, more sophisticated cytogenetic techniques have made it possible to detect smaller chromosomal anomalies that may have gone undetected in earlier days. Therefore, studies that excluded children with major chromosomal anomalies may unknowingly have included children with chromosomal anomalies that less sensitive techniques could not detect. In the future, it might be indicated to re-examine those children who were diagnosed earlier as

having a normal karyotype, both clinically by a clinical geneticist, as well as by using more detailed techniques, such as array-based Comparative Genomic Hybridization[42,43].

In summary, this study describes phenotypes of patients with EA/TEF and full or partial VACTERL association treated in two large pediatric surgical centers. Their clinical data are well-documented in comprehensive databases. Importantly, this study shows that almost 71% of patients display non-VACTERL-type congenital anomalies in addition to defects of the VACTERL spectrum. We believe this study gives a good overview of the spectrum of anomalies seen in these patients and that it adds to the knowledge of the clinical characteristics displayed by these patients.

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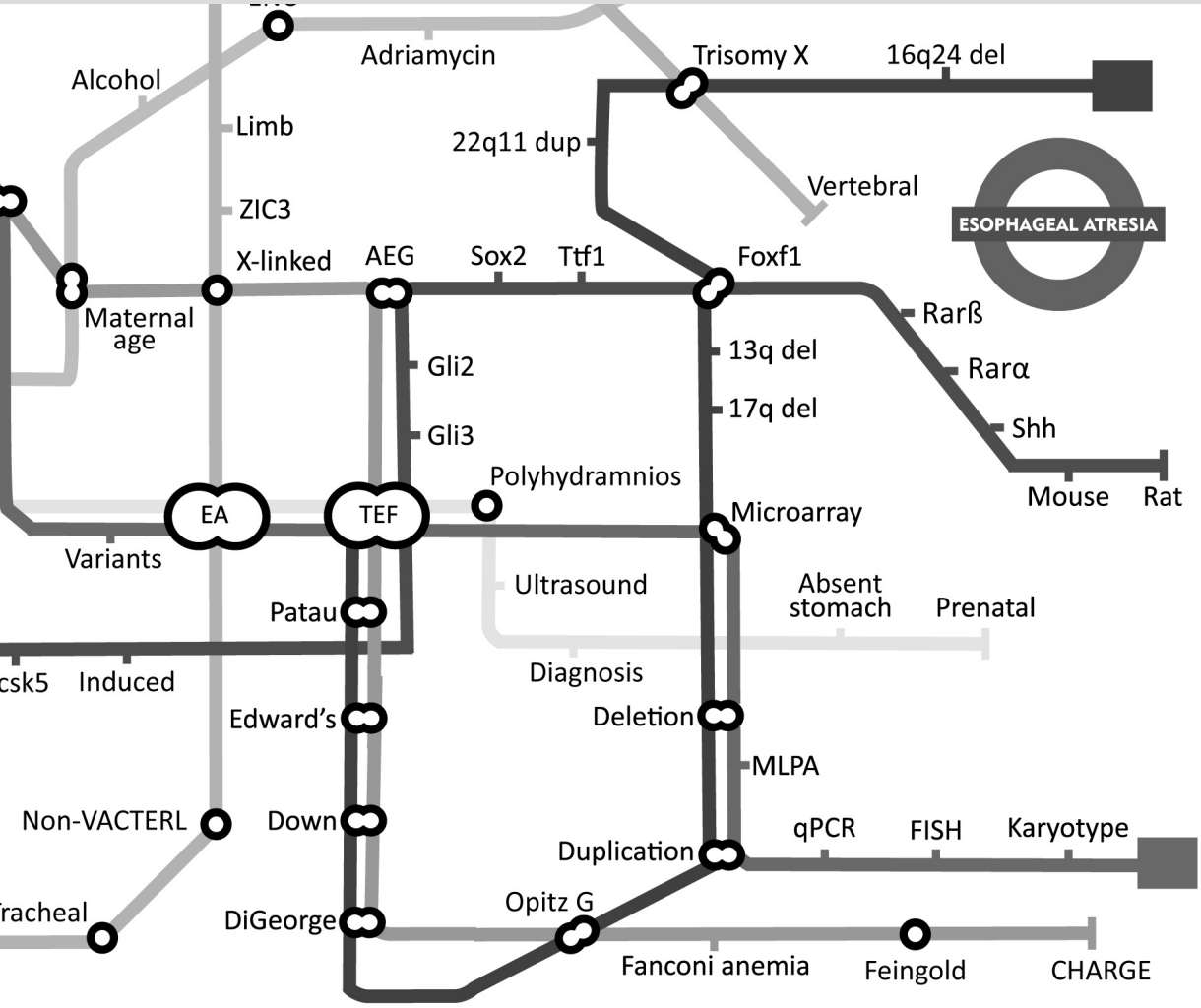
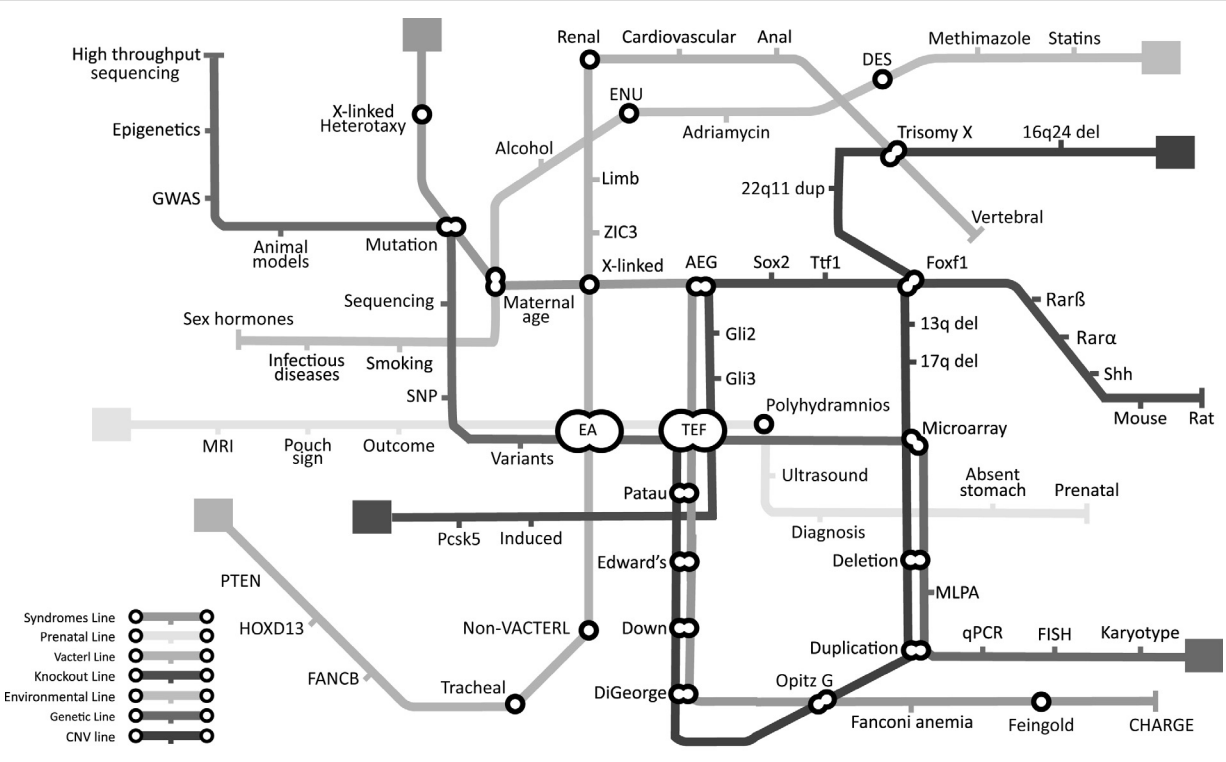
# PART III

## Molecular-Genetic Studies in Patients with Esophageal Atresia



# CHAPTER 4

## Familial Esophageal Atresia and Anorectal Malformation; a Causative Role for 22q11 Microduplication or a Chance Occurrence?




**Adapted from:**

**E.M. de Jong**, P.J. Poddighe, C.E.J. Sloots, D. Tibboel, A. de Klein, A.S. Brooks. Familial esophageal atresia and anorectal malformation; a causative role for 22q11 microduplication or a chance occurrence? *Submitted*

## Abstract

**Background: E**sophageal **a**tresia (EA) is a life threatening congenital anomaly that occurs in one in every 3,500 live-births. Little is known about possible underlying genetic factors. Familial occurrence of EA is extremely rare.

**Case report:** EA and anorectal malformation (ARM) were observed in a boy and his maternal uncle. The proband had VACTERL associated anomalies and mild dysmorphisms.

**Methods:** MLPA and microarray analysis were used to identify the cause for familial EA combined with ARM.

**Results:** This revealed a maternally inherited 22q11 microduplication in the proband. The 22q11 microduplication was not found in the mother's brother, who had the same phenotype as the proband.

**Conclusions:** This is the first case of EA and ARM associated with an inherited 22q11 microduplication syndrome. However, the 22q11 microduplication syndrome does not co-segregate with the EA/ARM phenotype in this family. Considering the incomplete penetrance, the 22q11 microduplication was not sufficient to cause the phenotype. Since a complex model with the contribution of two or more loci cannot be discarded, we postulate that additional genes are involved in the etiology of EA and ARM in this family.

## Introduction

**E**sophageal **a**tresia (EA) with or without **t**racheo-**e**sophageal **f**istula (TEF) and anorectal malformation (ARM) are common congenital birth defects, with estimated incidences of 1 in 3,500 and 1 in 5,000 live-born infants, respectively. They are often associated with other congenital malformations, fitting the so-called VACTERL association, including vertebral, cardiovascular, renal and limb abnormalities[1]. Both are thought to be multifactorial complex diseases with involvement of genetic and environmental factors. Either can be part of defined monogenic syndromes for which the underlying genes have been elucidated[2,3]. In addition, EA and ARM are also associated with cytogenetic abnormalities, including trisomies of chromosome 13, 18 and 21, and with deletions of different chromosomal loci, such as 22q11, 13q, 17q21.3-q24.2, and 7q36[3-5]. Familial occurrence of EA/TEF is extremely rare and only a few case reports have been published[6].



The plethora of syndromes and chromosomal aberrations associated with EA or ARM illustrates that these congenital malformations are genetically heterogeneous. A single causative gene has not been found, neither in isolated nor in patients with VACTERL association[1,3,4]. This might well be due to very slight individual effects of the susceptibility genes. High-resolution techniques, such as comparative genomic hybridization (CGH) or single nucleotide polymorphism (SNP) arrays, might be helpful to identify new genes in this respect. Using such techniques, we studied a family in which two male patients presented with combined EA/TEF and ARM. The proband had an inherited 22q11.2 microduplication, not segregating with the phenotype in the maternal uncle.

The 22q11.2 microduplication syndrome is caused by nonallelic homologous recombination between region-specific low-copy repeats (LCR-22s) found in the 22q11.2 region[7-9]. Such rearrangements also cause other genomic disorders in this region, e.g. the 22q11.2 deletion syndrome (DiGeorge/velocardiofacial syndrome [MIM 188400; MIM 192430][7]) and the Cat-eye syndrome [MIM 115470][10]. The 22q11.2 microduplication syndrome shows inheritance with variable phenotypes, but also *de novo* cases have been described[11,12]. The mode of inheritance is typically from a parent with a near-normal phenotype, mild cognitive deficits or mild dysmorphic features[13,14].

## Case report

The index patient (Figure 1A) was born at 37 6/7 weeks by spontaneous vaginal delivery following an uncomplicated pregnancy; the Apgar score was 8/10/10 after respectively 1, 5

and 10 minutes. Birth weight was 2280 grams (10th centile). Physical examination revealed a broad spectrum of anomalies, fitting the VACTERL association, described in Table 1. Within two days postnatally, the type C esophageal atresia was reconstructed with a primary anastomosis and a colostomy was created. The patient's mild dysmorphic features included hypertelorism and small ears without pits or tags (Figure 1B). The boy is 1 year of age at the time of writing; developmental milestones for the first year were reached. Motor and cognitive development were normal. Neurological examination at 11 months of age revealed some aberrations: hypertonic muscles in the legs with a slightly hyperactive symmetric muscle reflex. In addition, the sitting balance was impaired.

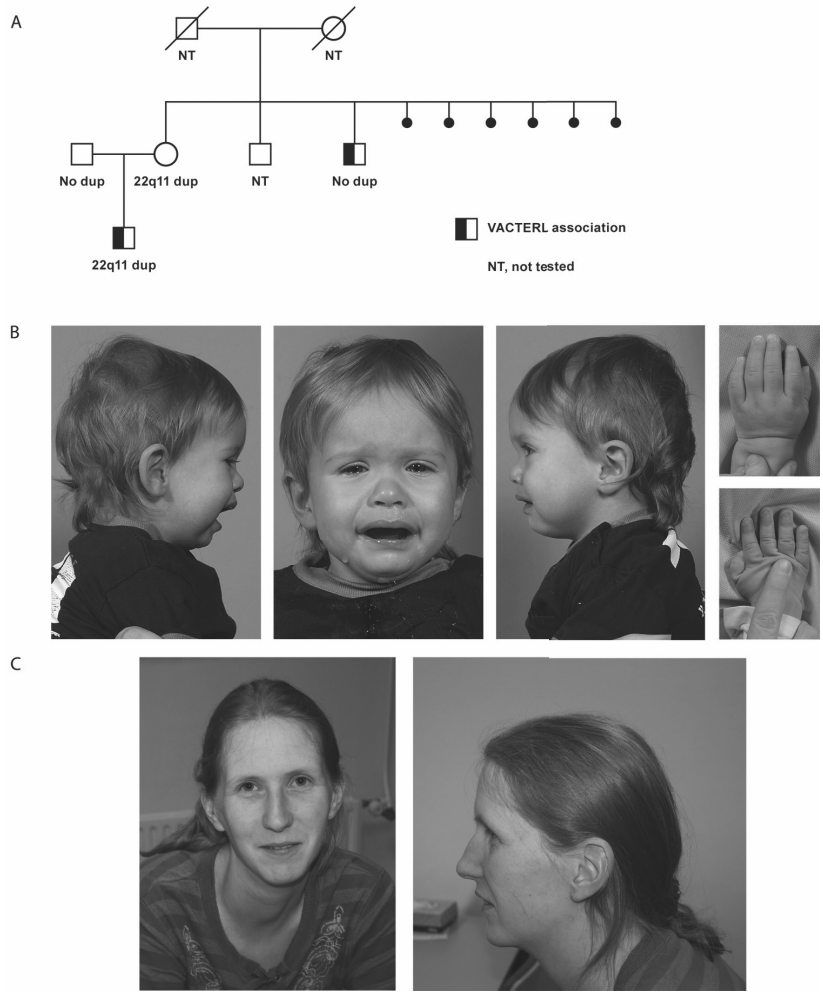



**Figure 1: Pedigree and photo's of EA/ARM family members**
*Color figures can be found in the appendix. See page 184*

The mother's height and weight were normal, and she had no congenital malformations. She showed mild dysmorphic features, i.e. deepset eyes, large, tubular noise, high palate, crowding of the teeth and low-set ears (Figure 1C). She had slender fingers, but no other abnormalities of the extremities. She had mild learning difficulties. The maternal grandmother had six miscarriages and had died at the age of 42 years after a CVA.

The mother's brother, now aged 23 years, was treated at our center; he was born with multiple congenital anomalies, summarized in Table 1. For his renal anomalies he was transplanted at the age of two years. No dysmorphic features were noted apart from synophrys and low-set ears. Formal IQ testing was never performed, but in childhood he had learning difficulties. He is now employed as help on a children's farm.



**Table 1: Overview of anomalies in the EA/ARM family with 22q11 microduplication syndrome**

| Anomalies | Proband * | Maternal uncle | Mother of proband * |
|---|---|---|---|
| Vertebral | Dysplastic S2<br>Absence of the rest of the sacrum | - | - |
| Anal | Anorectal malformation type recto-urethral-bulbar fistula | Anorectal malformation type recto-urethral-bulbar fistula | - |
| Cardiovascular | Multiple VSDs | - | - |
| Tracheo-esophageal | Esophageal atresia with tracheo-esophageal fistula | Esophageal atresia with tracheo-esophageal fistula | - |
| Renal | Small left kidney, including ectasia of the pyelum<br>Echo dense renal cortex | Agenesis of the left kidney<br>Dysplastic right kidney | - |
| Limb | Hypoplastic right thumb with radial deviation of the distal phalanx | - | Slender fingers |
| Dysmorphic features | Hypertelorism<br>Small ears | Synophrys<br>Low-set ears | Deepset eyes<br>Large and tubular nose<br>High palate<br>Crowding of the teeth<br>Low-set ears |
| Other | - | Double urethra<br>Hypospadias<br>Dyplastic hips | - |

*, confirmed 22q11 microduplication of 1.5 Mb; VSD, ventricular septal defect

The patient's father appeared normal with a negative family history for congenital and/or chromosomal aberrations.

The combination of congenital anomalies in the proband, the familial occurrence, and the observation of the family history of recurrent miscarriages raised the suspicion of a chromosomal abnormality. Laboratory studies, including karyotyping, FISH 22q11, MLPA 22q11 and microarray studies, were performed. The results are reported here.

## Methods

### *Cytogenetic analysis*

Chromosome banding analyses of the boy, his parents and his maternal uncle were done by standard protocols.

### *Multiplex Ligation-Dependent Probe Amplification (MLPA) analysis*

Genomic DNA was extracted from peripheral blood lymphocytes using standard procedures. MLPA using the SALSA MLPA kit P250, (MRC-Holland, Amsterdam, The Netherlands)[15] was performed according to the manufacturer's protocol; PCR products were separated and quantified as previously described[16]. Genemarker 1.51 (SoftGenetics, LLC, State College, PA, USA) was used for data analysis.

### *Microarray analysis*

High-resolution analyses were performed using the 250K Nsp1 array (Affymetrix®) according to the manufacturer's instructions (http://www.affymetrix.com). The microarrays were imaged by the Affymetrix® GCS3000-G7 scanner. Genotype calls and probe intensities were extracted with the Affymetrix® GeneChip Command Console (AGCC) software (version 1.1.). Genotype- and copy number variations (CNV)-analyses were performed using the BRLMM and CNAT 4.0.1 algorithms. CNVs were visualized as log2 ratios, detected by comparison to a common reference set of 40 CEU samples from the HapMap project (www.hapmap.org/downloads/raw_data/500K/). The segment-reporting tool filter was set to a minimum of three aberrant markers per segment. In addition, we performed a second CNV analysis, using an in-house control sample for detection of CNVs smaller than 100 Kb in size. Visualization was done with the Nexus® Copy Number software package version 4.0.

### *Fluorescent in situ hybridization (FISH)*

To validate MLPA and microarray findings, FISH experiments were performed according to standard protocols. The BAC clones were selected from the UCSC genome browser (UC Santa Cruz), purchased at BACPAC resources (Oakland) and labeled (Random Prime Labelling

System, Invitrogen) with Bio-16-UTP or Dig-11-dUTP (Roche). Probes were validated on control metaphases and used to examine the 22q11 region (cosmid M51, DNA marker locus D22S183; cosmid 122B5, DNA marker locus D22S111) and a 22qtel probe (CTD-3018K1) as control probe.

## Results

Karyotyping was normal in the four persons tested. The MLPA Salsa P250 kit revealed an enhanced signal for nine probes, indicating a duplication of the region between CLTCL1 and CGCR8 on 22q11 in the proband and his mother (Figure 2A). MLPA, using the DNA of the proband's father and maternal uncle, revealed no aberrations. FISH showed an amplified hybridization signal of M51 on one chromosome 22q11.2, confirmed in 20 interphase nuclei, in the proband and his mother (Figure 2B). Cosmid 122B5 showed a normal signal distribution on both chromosomes 22q11.2. The 22q11 microduplication was not found in the uncle.

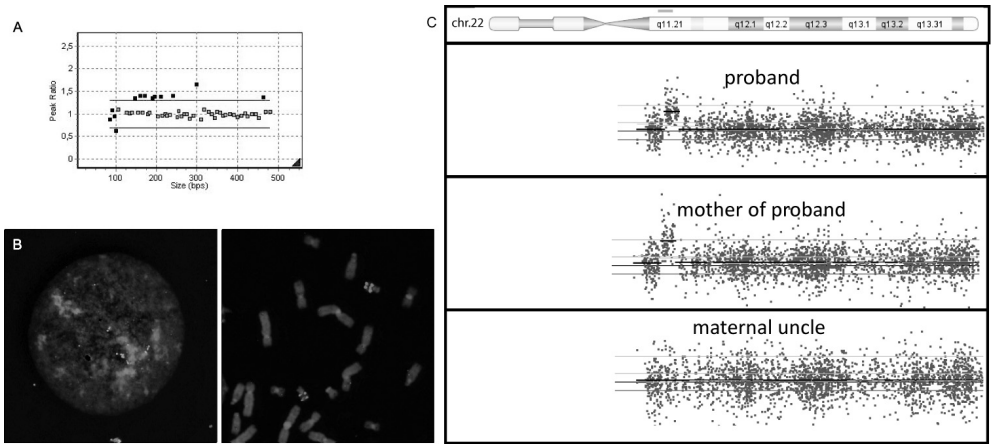



**Figure 2: Results of the laboratory studies**

A The MLPA Salsa P250 kit revealed an enhanced signal for nine probes, indicating a duplication of the region between CLTCL1 and CGCR8 on 22q11 in the index patient and his mother.

B FISH analysis revealed a duplicated signal of the cosmid M51 (22q11; red) and a normal signal of cosmid 122B5 (22qtel; green).

C Microarray studies confirmed the microduplication in the index patient and his mother.

*Color figures can be found in the appendix. See page 185*

To determine the boundaries of the duplication and to identify other possible phenotype-affecting CNV, we performed a genome wide Affymetrix® 250K Nsp1 microarray in the boy, his mother, and his uncle. This confirmed the MLPA results in that it revealed an identical interstitial duplication of the 22q11.2 chromosomal region in the proband and his mother, but

not in the uncle (Figure 2C). The proximal breakpoint was at position chr22:17,147,764; the distal breakpoint was at position chr22:18,648,997 (UCSC Genome Browser, http://genome.ucsc.edu; Build 36.1; accessed November 2008), resulting in a 1.5 Mb duplication from LCR22-A (22-2) through LCR22-B (22-3a). The region included 28 genes. A comparison of the microarray data for additional copy number variations revealed no additional possible pathogenic CNVs.

## Discussion

In this communication we describe a family in which the proband and his maternal uncle had EA and ARM, associated with a 22q11 microduplication syndrome in the proband. The initial working clinical diagnosis for the proband was a 22q11 microdeletion or Cat-eye syndrome, in view of the cardiac phenotype and the ARM. However, the boy showed a 1.5 Mb microduplication on chromosomal locus 22q11.2, inherited from his mother. Our study confirms the wide phenotypic variation (Table 1)[9], given that the boy's mother showed only mild dysmorphic features, combined with mild learning disabilities.

The clinical phenotype of patients with the 22q11.2 microduplication syndrome was recently reviewed by different authors[13,14,17]. Clearly, 22q11 microduplications are subject to incomplete penetrance and variable expressivity. The reported phenotypic variation ranges from no abnormality or mild learning disabilities[18,19] to severe mental retardation and severe congenital abnormalities, including non-specific congenital heart defects, velopharyngeal insufficiency, hearing loss, postnatal growth deficiency, urogenital anomalies, and dysmorphic facial features[13,17]. The phenotype driven detection approach carries the risk of bias, since there is a clear overlap in phenotype with the 22q11 deletion syndrome. The described phenotypes are non-specific and so far it is not known to what extent the 22q11.2 duplication is responsible for these features.

Most patients describe duplications of 3 Mb and higher, but we found a small 1.5 Mb duplication. The variable size and gene content of the duplications of the 22q11.2 region may contribute to the phenotypic differences. However, the VACTERL associated anomalies of our proband have not yet been described in the clinical spectrum of the 1.5 Mb duplications[14]. A search in publicly available Internet databases, such as DECIPHER and ECARUCA (https://decipher.sanger.ac.uk/; version 4.4[20]; http://www.ecaruca.net/[21]; accessed 20 February 2010) revealed no other cases of overlapping duplications with a comparable EA/ARM phenotype.

The variability of these 22q11 duplications raises the question whether they are non-pathogenic CNVs or pathogenic variants with incomplete penetrance. Indeed, microduplications of 22q11 have been reported in cohorts of unaffected individuals (n = 6)[22], and in a cohort

of patients with developmental disabilities screened for CNVs (n = 18)[11]. Different causes have been proposed for the variable expressivity and incomplete penetrance in the 22q11 microduplication syndrome; i.e. other genetic mutations elsewhere in the genome, compensation of other genes or downregulation of gene targets of the duplicated genes, epigenetic factors, or a functional SNP on the non-rearranged chromosome could be affected by the genomic rearrangement[14,19,23]. In addition, copy number variation itself could explain reduced penetrance of disease-causing mutations; a dominant loss-of-function mutation might be rescued by a gain in copy number, resulting in increased gene expression[24]. This hypothesis was recently validated when a compensatory microduplication on 22q11 rescued the decreased gene expression of a similar microdeletion on the sister allele, in a parent of a patient with DiGeorge syndrome[25]. Furthermore, a two-hit model was proposed in children with severe developmental delay, in which one microdeletion predisposes to neuropsychiatric phenotypes, exacerbated in association with other large deletions or duplications[26]. Therefore, we might deduce that there are compensatory copy number variations in the mother in our study.



The duplicated segment LCR22-A through LCR22-B (1.5 Mb) maps 28 genes, including the most important candidate gene *TBX1*. *TBX1* has been proven to be causal to several congenital birth defects seen in the 22q11 microdeletion syndrome and this deletion was described in some patients with EA or ARM[5,27,28]. Gain of function mutations of *TBX1* were described in patients with mental retardation and Shprintzen syndrome, with a comparable level of gene expression as in the microduplication syndrome[29,30]. Caterino *et al.* found that overexpression of *TBX1* down regulates proteins involved in retinoic acid metabolism, which is a main regulator of many developmental processes, including heart and foregut development[31]. *TBX1* is dosage sensitive and this may affect developmental pathways in humans, contributing in both the microdeletion and microduplication syndrome.

Another attractive hypothesis for the combined phenotype in two male patients in this family would be a CNV located on the X-chromosome, which would fit with the milder phenotype in the mother. Yet the microarrays did not identify additional microdeletion or microduplication on the X-chromosome. However, very small CNVs – below 100kb – might have escaped detection by the array platform used. Alternatively, this family represents a rare monogenic inherited X-linked recessive disorder that cannot be tackled by SNP microarrays. VACTERL anomalies are present in both the patient and his maternal uncle and mutations in *FANCB* or *MID1* could also be responsible for X-linked Fanconi anemia or X-linked Opitz syndrome. It is expected, however, that next generation sequencing techniques with the

ultimate resolution to screen the human genome at base pair level, e.g. exome sequencing, will become instrumental in identifying possible underlying gene defects[32].

Our proband's complex phenotype in combination with the familial occurrence of his congenital malformations raised the suspicion of a chromosomal abnormality. We used MLPA and microarrays to pick up the 22q11 microduplication. Furthermore, our results confirmed that specific MLPA is a reliable method in detecting different sizes of the duplications[15]. However, considering the incomplete penetrance in this family, the 22q11 microduplication may be necessary, but not sufficient to cause the phenotype. Assuming that the 22q11 microduplication could contribute to the phenotype and does not represent a chance occurrence, we hypothesized the existence of other genes and/or genomic variants outside the duplicated 22q11 region in this family. Whole genome microarrays thus excluded additional copy number variations, but small variants may have escaped detection with these microarrays. Nevertheless, 22q11 microduplication syndrome clearly cannot solely explain the VACTERL association in this family since it does not segregate with the phenotype.

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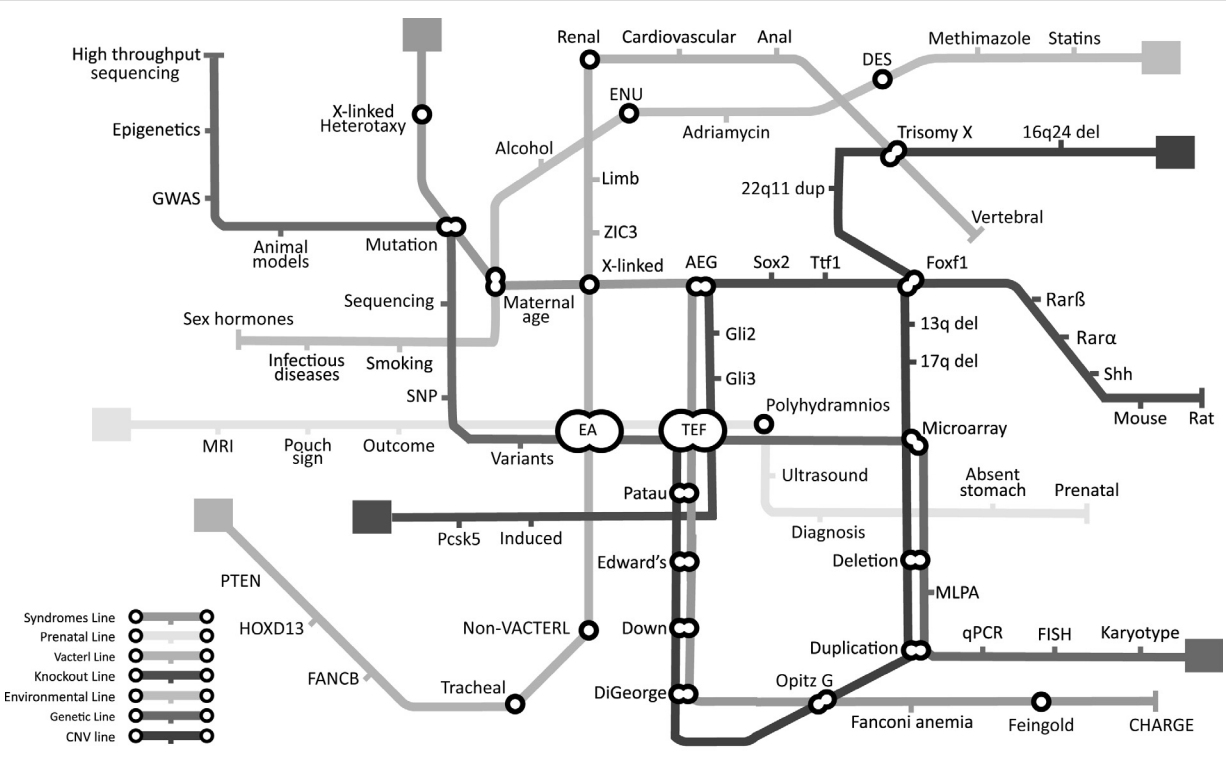
# CHAPTER 5

## Congenital Malformations of the Gastro-Intestinal Tract and Triple X Syndrome




**de Jong EM**, Barakat TS, Eussen BH, D'haene B, De Baere E, Poddighe PJ, Galjaard RJ, Gribnau J, Brooks AS, Tibboel D ,De Klein A. Congenital malformations of the gastro-intestinal tract in Triple X syndrome. *Submitted.*

## Abstract

**Introduction:** **E**sophageal **a**tresia (EA) with or without **t**racheo-**e**sophageal **f**istula (TEF) is a relatively common birth defect with an incidence of 1:3,500 births. Over half of the patients show other major anomalies, predominantly VACTERL associated anomalies (**V**ertebral, **A**nal, **C**ardiovascular, **T**racheo-**E**sophageal, **R**enal and **L**imb). Both genetic and environmental factors are thought to play causative roles. We sought to identify genes or loci involved in EA/TEF.

**Methods:** Retrospectively, 269 karyotypes of EA/TEF patients were evaluated and telomere-MLPA was used to identify subtelomeric aberrations in our EA/TEF cohort. Genome-wide copy number profiling on SNP arrays was done in selected patients. In addition we investigated X-chromosome inactivation (XCI) patterns and mode of inheritance of the aberrations.

**Results:** Three patients had a 47,XXX karyotype; two other patients showed inherited duplications of the *SHOX* gene. The latter showed two or more anomalies within the VACTERL spectrum apart from EA/TEF. The three patients with a 47,XXX karyotype were all small for gestational age at birth (<P5). One of these displayed VACTERL anomalies, while the other two patients had in addition to EA/TEF mild dysmorphic features and a ventricular septal defect. Patterns of X-chromosome inactivation (XCI) were normal, leaving one X-chromosome active. In all three patients the extra X was maternally inherited and the inactivation pattern was random in relation to the parental X.

**Conclusions:** Until now, Triple X syndrome is rarely described in patients with EA/TEF and no inherited duplications of the *SHOX* gene were reported so far in patients with EA/TEF. Since normal patterns of XCI were seen, overexpression of X-linked genes that escape XCI, e.g. the *SHOX* gene, could influence developmental pathways.

## Introduction

**E**sophageal **a**tresia (EA) with or without **t**racheo-**e**sophageal **f**istula (TEF) is a relatively common birth defect occurring in approximately 1:3,500 births[1]. There may be causal involvement of multiple genetic and environmental factors. Associated anomalies occur in over 50% of patients; these are predominantly VACTERL associated anomalies (**V**ertebral, **A**nal, **C**ardiovascular, **T**racheo-**E**sophageal, **R**enal and **L**imb). Chromosomal anomalies or point mutations are found in 10% of EA/TEF cases[2]. EA/TEF is a variable feature in Feingold, CHARGE, Opitz G, and **A**nophthalmia-**E**sophageal-**G**enital (AEG) syndrome, and in Fanconi anemia[3], but may also occur in syndromes caused by X-linked mutations, such as Opitz G syndrome (*MID1)*[4] and VACTERL association with hydrocephalus (*FANCB)*[5]. In 90% of EA/TEF patients, the cause is unknown.

Many X-linked syndromes have been described in patients with congenital malformations, such as sex chromosome aneuploidies (e.g. Klinefelter and Turner syndrome)[6,7] and mutations in X-linked genes that cause monogenic diseases, e.g. X-linked mental retardation in Fragile X syndrome (*FMR1*)[8]. However, sex chromosome aneuploidies or copy number aberrations on the X-chromosome are rare in patients with EA/TEF[9-11].

The triple X karyotype, first described by Jacobs *et al.*[12], occurs in 1:1,000 girls[13]. In 90% of cases, the additional X-chromosome is the result of a maternal meiotic I error[14]. The incidence of non-disjunction errors increases with maternal age[15]. Girls with the Triple X syndrome have a lower birth weight, more accelerated growth until puberty, long legs, more behavioral problems and more psychiatric disorders than other girls[16]. A direct relation between gastro-intestinal anomalies was not yet established, however the literature describes at least six cases with gastro-intestinal anomalies and Triple X syndrome[17].

Overexpression of the Short stature HOmeoboX-containing gene (*SHOX*) is rare. Duplications of *SHOX*, located within the pseudoautosomal region (PAR1) of X(p) and Y(p) are described, but with no distinct phenotype[18,19]. *SHOX* duplications in relation to gastro-intestinal anomalies have not been described so far.

This study consisted of a retrospective evaluation of cytogenetic abnormalities in our institution's EA/TEF cohort (n=321). Three patients had a triple X karyotype. Additional molecular-genetic studies with telomere-MLPA and SNP arrays found *SHOX* duplications in two other patients. We reviewed the literature for patients with Triple X syndrome in relation to congenital gastro-intestinal malformations. We hypothesized that genes on X and/or genes that escape chromosome X inactivation could influence developmental pathways, such as in foregut development.

## Methods

### *Patients*

From 1988 onwards, all patients with EA/TEF, admitted to the department of Pediatric Surgery, Erasmus MC, Rotterdam, The Netherlands were included (n=321). Pregnancy, clinical and follow-up data were collected and stored in a comprehensive database. Available DNA and cell lines of patients (n=180) and parents were collected and stored. Patients with defined syndromes or point mutations in certain genes had been excluded from further molecular-genetic evaluation. From 1988 onwards, the database of the department of Clinical Genetics, Erasmus MC, The Netherlands, was searched for triple X karyotypes and confirmed *SHOX* duplications at the departments of prenatal and postnatal diagnostics. Phenotypes of patients were extracted from medical charts available at the Department of Clinical Genetics.

### *Cytogenetic evaluation*

Routine cytogenetic screening was performed for all patients with major congenital anomalies; karyotyping was performed according to standard protocols on lymphocytes from peripheral blood cultures. From 1988 onwards, 269 karyotypes were available.

### *Statistical analysis*

Differences in two proportions were tested with the Pearson's chi-square ($\chi 2$) test, reported with a 95% confidence interval (CI), performed in SPSS 15.0.

### *Multiplex ligation-dependent probe amplification (MLPA) and quantitative PCR*

Subtelomeric aberrations were tested with MLPA analysis, using the P036E1 and P070A2 Salsa telomere kit (MRC Holland, Amsterdam, The Netherlands). Reactions were performed according to the manufacturer's protocol; PCR products were separated and quantified as previously described[20]. Genemarker 1.6 (SoftGenetics, LLC, State College, PA, USA) was used for data analysis. Copy number profiling of the *SHOX* region was done with a newly developed qPCR assay, with 11 amplicons within *SHOX* and the PAR1 region[21].

### *Fluorescent in situ hybridization (FISH)*

To confirm the results, BAC clones for Xp22 and Xq25 were selected from the University of Santa Cruz (UCSC) genome browser and ordered from BACPAC Resources. After isolation of the BAC DNA, the probes were labeled and used for FISH on chromosome preparations from patients and parents, according to standard protocols[22].

### *RNA-FISH analysis and immunocytochemistry of human cell lines*

RNA-FISH and immunocytochemistry were performed on fibroblast cell lines (confluency reached >90%) and EBV-transformed lymphocytes, as previously described by Jonkers *et al.*[23].

### *HUMARA analysis*

To determine the parental origin and the methylation status of the additional X-chromosome, we used the HUMARA assay (**H**uman **M**ethylation of the **A**ndrogen **R**eceptor **A**ssay)[24] in the triple X patients and their parents; 40ng of genomic DNA was double digested with DdeI and HpaII (New England Biolabs) at 37°C in an overnight reaction. 1µl of reaction product serves as a template for PCR with Phusion polymerase (Finnzymes), using the primers GCTGTGAAGGTTGCTGTTCCTCAT and TCCAGAATCTGTTCCAGAGCGTGC. PCR products were analyzed by gel electrophoresis on an 8% PAGE gel, runned overnight at 66V at 4°C, stained with ethidium bromide and scanned using a Typhoon scanner (GE Healthcare). Images were further analyzed using Image Quant software (GE Healthcare).



### *Microarrays*

To further characterize possible additional chromosomal aberrations in the patients with Triple X syndrome, copy number was analyzed using Affymetrix® single nucleotide polymorphism (SNP) genotyping microarrays. DNA extracted from blood was genotyped on Affymetrix® 250k Nsp (Affymetrix®, Santa Clara, CA). The copy number analysis package within CNAT 4.0 (Chromosome Copy Number Analysis Tool, http://www.affymetrix.com/) was used to analyze the signal intensity values of SNPs. Samples were normalized by quantile normalization on perfect match probes, following allele summarization utilizing the BRLMM software component within GTYPE (available from http://www.affymetrix.com/), per the default CNAT 4.0 protocol.

The HumanCytoSNP-12 chip (Illumina®, San Diego, CA, USA) was used in the cases with *SHOX* duplications and their parents, for better coverage of the PAR1 region. All procedures were done according to the manufacturer's protocol. All products were scanned with the Illumina Beadstation scanner and raw data were uploaded in Beadstudio v2.0 genotyping software (Illumina®, San Diego, CA, USA) for further analysis.

Visualization of all microarrays was done with the Nexus Copy Number software package (V4; BioDiscovery® Inc., El Segundo, CA, USA).

## Results

*Triple X syndrome*

Karyotyping had been performed for 269 of the 321 patients of our cohort, as systematic cytogenetic follow-up of patients with congenital anomalies was not carried out from 1988-1998. A triple X karyotype was identified in three patients (Figure 1A). The odds ratio for Triple X syndrome in our EA/TEF cohort is 11.3, (95% CI= 3.6 to 35.2). All three were small for gestational age at birth (<5th centile). One patient displayed all VACTERL anomalies and additional genito-urinary anomalies, while the other two patients had mild dysmorphic features and a ventricular septal defect in addition to EA/TEF, respectively (patients 1-3, Table 1). At time of delivery the mothers were aged under 35. In addition, the database of the Department of Clinical Genetics, Erasmus MC, yielded 59 non-mosaic 47,XXX karyotypes since 1988; 29 had been detected prenatally and 30 postnatally (apart from the above three patients). Indications (not mutually exclusive) for prenatal karyotyping were: maternal age above >35 years (n=25), congenital malformations on ultrasound (n=4), increased risk for Down syndrome on first trimester screening ultrasound (n=2), increase in fetal bowel echogenicity on ultrasound (n=1), and congenital anomalies in an earlier pregnancy (n=3). One screening for the reason of increased maternal age concerned a twin pregnancy of which one sib had a 47,XXX karyotype. Postnatally, patients were karyotyped based on the following indications: suspicion of Fragile X syndrome (n=5), mental retardation (n=5), multiple congenital anomalies (n=4), combined mental retardation and multiple congenital anomalies (n=3), repeating spontaneous abortions in the index (n=4), failure to thrive (n=4), a chromosomal abnormality in the family (n=3), suspicion of trisomy 21 (n=1), and a possible chromosomal aberration in juvenile systemic lupus erythematosus (n=1).

Postnatal follow-up of thirteen pregnancies was not documented. Seven pregnancies were terminated, in one case because the fetus showed anencephaly. Congenital malformations had been documented for three of the nine live-borns; a neural tube defect, hygroma colli with generalized edema, and osteogenesis imperfecta, respectively. A review of the medical charts of the postnatally diagnosed patients identified at least four patients with congenital heart defects. Most patients had psychomotor retardation and psychiatric problems.

RNA-FISH and immunocytochemistry were performed to assess the X inactivation status of cell lines derived from the 47,XXX patients in our cohort. This RNA-FISH revealed two *XIST* clouds in almost every fibroblast and lymphocyte cells (>95% of the nuclei), co-localizing with an area of low level COT-1 expression (Figure 2A). Immunocytochemistry of 47,XXX fibroblasts detected enrichment of the facultative heterochromatin markers H3K27me3 and MacroH2A1

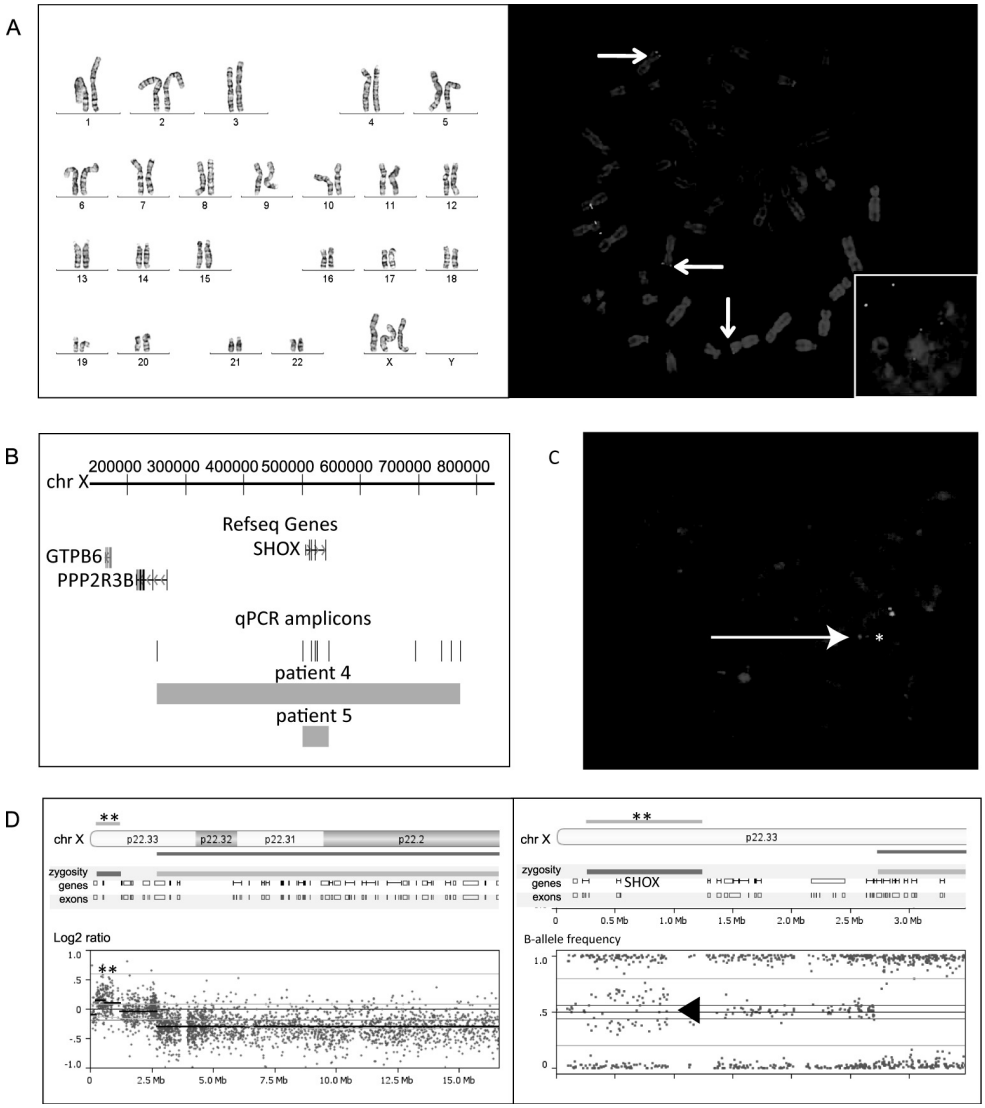



**Figure 1: Triple X syndrome and *SHOX* aberrations in patients with EA/TEF**

1A 47,XXX karyotype confirmed with FISH. BAC probe on Xp22 (RP11-800K15; red)

1B Validation of *SHOX* duplications with *SHOX* specific qPCR in both patients. The green lines indicate the length of the duplications, with a larger duplication in patient 4 (amplicon 1-13) and a specifc *SHOX* duplication in patient 5 (amplicons 2-8).

1C *SHOX d*uplication confirmed with FISH in patient 5. Probe RP11-800K15 on Xp22 (red), covering the *SHOX* gene, shows a duplicated signal (*) and a control probe RP11-49N19 on Xq25 (green).

1D The HumanCytoSNP-12 chip (Illumina®, San Diego, CA, USA) showing an interstitial duplication of the PAR1 region (base pair position chrX:248,968-1,229,976), including the SHOX gene, in the father of patient 4. In the left panel, the elevated log2 ratio indicates the duplicated segment (green bar, **) and the B-allele frequencies change in ratio indicated in the enlarged right panel (purple bar). *Color figures can be found in the appendix. See page 185*

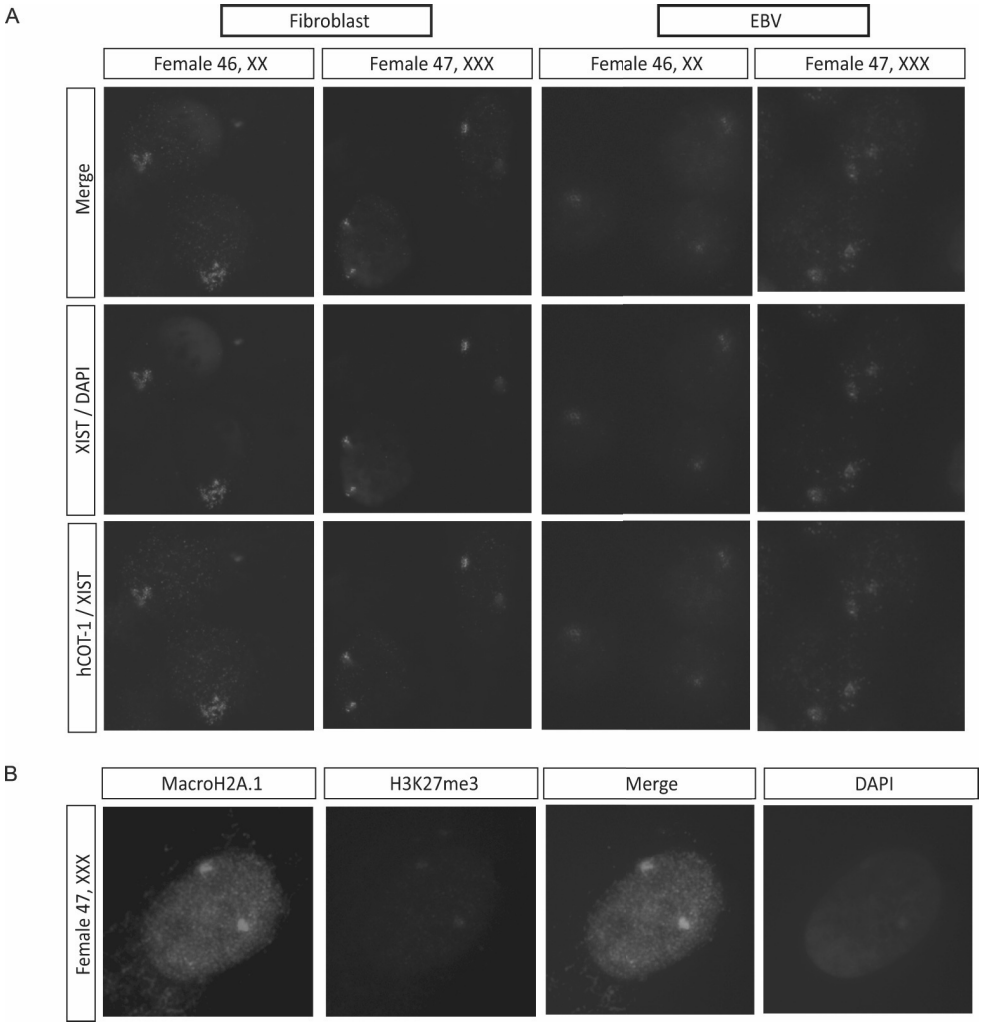



**Figure 2: Chromosome X inactivation studies in triple X patients**

2A RNA-FISH analysis detecting *XIST* and COT-1 RNA. *XIST* RNA was detected using a digeoxin-labelled cDNA probe (FITC), and a biotin-labelled COT-1 DNA probe detected expression of repetitive sequences (Rhodamine). In 47, XXX patient cells two *XIST* clouds and COT-1 holes were detected in the majority of the cells (> 95% of the nuclei, n >100). Nuclei were counterstained with DAPI.

2B Immunocytochemistry detecting H3K27me3 (Rhodamine) and MacroH2A1 (FITC). Enrichment of H3K27me3 and MacroH2A1 was found on two X-chromosomes in 47, XXX fibroblast cell lines, co-localizing with DAPI-dense Barr bodies, indicative of two inactive X-chromosomes. Nuclei are counterstained with DAPI. *Color figures can be found in the appendix. See page 188*

on two X-chromosomes, co-localizing with the DAPI-dense Barr-bodies (Figure 2B). HUMARA analysis confirmed a maternal origin of the additional X-chromosome in all three patients. In both the patients and their mothers random XCI was observed, with no skewed preference of inactivation of a particular X-chromosome (data not shown). Additional SNP array analysis (Affymetrix® 250k Nsp) performed to exclude copy number variations elsewhere in the genome, did not reveal additional possible pathogenic copy number variations in any of the three patients.

### *SHOX duplications*

DNA of 180 patients was screened with telomere-MLPA. The results indicated *SHOX* duplications in two boys (patients 4 and 5). The twin sib of patient 4 was spontaneously aborted in the third month of gestation. His mother was diagnosed with Goldenhar syndrome and two relatives on the mother's side have thumb anomalies. The boy was small for gestational age at birth (<2SD) and had associated cardiovascular, renal, limb and genital anomalies (Table 1). Patient 5 was also small for gestational age at birth. He displayed associated cardiac and limb anomalies. The database of the Department of Clinical Genetics, Erasmus MC, yielded no additional *SHOX* duplications with a congenital phenotype.



Both *SHOX* duplications were validated with a *SHOX*-specific qPCR (Figure 1B). Validation confirmed the duplications of the *SHOX* gene and the amplicons in the PAR1 region in patient 4, as being inherited from his father. Cultured lymphocytes for FISH validation were not available. Validation also confirmed the *SHOX* gene duplication in patient 5, as being inherited from his mother. FISH also confirmed the duplication of the *SHOX* gene on Xp22 in this patient (Figure 1C). The father and mother in question had no EA/TEF. To exclude additional copy number variations of other regions in the genome, both patients and their parents were screened with genome-wide microarrays (HumanCytoSNP-12 chip V2, Illumina®). These arrays revealed multiple copy number polymorphisms in both the patients and their parents. However, closer examination of these regions did not indicate potential pathogenic copy number variations.

To delineate the duplicated segment in patient 5 and his father, we focused on the CNVs in the X/Yp PAR1 region. Although the exact boundaries of the duplication could not be determined in the patients' sample, due to diminished quality of the array, the duplicated segment could be delineated in the father to a interstitial duplication of PAR1 of 981Kb (base pair position chrX:248,968-1,229,976) (Figure 1D).

**Table 1: Congenital anomalies of patients with EA/TEF and an anomaly on the X-chromosome**

| Pt no | Karyotype | MLPA results | Clinical features |
|---|---|---|---|
| 1 | 47,XXX | X/Yp SHOX enh | Absent sacrum<br>Anal atresia<br>Pulmonary stenosis<br>EA + TEF<br>Vesico-urethral reflux; urethral atresia<br>Absent thumbs<br>Cloacal malformation; abnormal labia; hydrometrocolpos |
| 2 | 47,XXX | X/Yp SHOX enh | TEF<br>Dysmorphic features |
| 3 | 47,XXX | X/Yp SHOX enh | EA + TEF<br>Ventricular septum defect<br>Thin fingers |
| 4 | 46,XY | X/Yp SHOX enh<br>*paternal inheritance* | Aberrant subclavia<br>EA + TEF<br>Horseshoe kidneys<br>Adducted thumbs; left thumb smaller than right<br>Hypospadias<br>Frontal bossing<br>Dysmorphic features |
| 5 | 46,XY | X/Yp SHOX enh<br>*maternal inheritance* | Atrial septum defect (type II)<br>EA + TEF<br>Proximal placement of thumbs |

EA, esophageal atresia; TEF, tracheo-esophageal fistula; enh, enhanced signal with P036E1 and P070A2

## Discussion

Congenital malformations and mental retardation syndromes have been linked to the X-chromosome. Our search for genes or loci involved in EA/TEF or other foregut-related anomalies identified five patients in our cohort with chromosome X/Yp aberrations; three with Triple X syndrome and two with inherited *SHOX* duplications.

The Triple X syndrome is usually a random finding in cytogenetic follow-up of pregnancies and in genetic screening of patients with mental retardation, psychiatric disorders and/or congenital malformations. A large proportion of triple X females have a subclinical phenotype. With an estimated occurrence of 1 in 1,000 females, many females are undiagnosed. In our cohort the incidence of Triple X syndrome is 11 times higher than that in the general population. Congenital anomalies associated with the Triple X syndrome described in case

**Table 2: Congenital malformations of the gastro-intestinal tract and/or foregut-related structures in Triple X syndrome**

| N | Genital | Urinary | Gastro-intestinal | V | A | C | TE | R | L | Other anomalies | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 3 | + | + | cloacal extrophy incl. imperforate anus, esophageal atresia + TEF | + | + | + | + | | + | dysmorphic features | *present study* |
| 1 | | | jejunal atresia | | | | | | | | ***33*** |
| 1 | | | duodenal atresia | | | | | | | | ***32*** |
| 1 | | | duodenal atresia* | | | + | | | | | ***17*** |
| 1 | | | omphalocele | | | | | | | Beckwith-Wiedemann syndrome | ***28*** |
| 1 | | | omphalocele | | | | | + | | | ***29*** |
| 1 | + | + | | | | | | + | | pulmonary hypoplasia, laryngeal atresia, craniofacial anomalies | ***50*** |
| 1 | | | ectopic anus | | + | | | | + | inferior coloboma, clinodactyly, dysmorphic features | ***34*** |
| 1 | + | + | cloacal extrophy incl. imperforate anus | | + | | | | | | ***30*** |
| 1 | + | + | cloacal extrophy incl. imperforate anus and rectoperineal fistula, colonic atresia, omphalocele | | + | | | + | | | ***31*** |
| 1 | + | + | imperforate anus, esophageal atresia + TEF | + | + | | + | + | | pulmonary hypoplasia, agenesis of gallbladder | *11* |

V, vertebral defects; A, anorectal malformations; C, cardiovascular anomalies; TE, esophageal atresia and/or tracheo-esophageal fistula; R, renal anomalies; L, limb malformations; *, duodenal atresia due to annular pancreas

reports, include anomalies of the urinary tract, genital anomalies and craniofacial anomalies, specifically a reduced head circumference and/or decreased brain volume[16,25-27]. Gastro-intestinal anomalies, including atresias of the esophagus, duodenum and jejunum, as well as omphalocele and anorectal malformations, have been reported sporadically[11,17,28-34]. All thirteen reported patients with gastro-intestinal and/or foregut-related anomalies are reviewed in Table 2. The patient described by Hoang *et al.* shows a similar phenotype compared to our patients; higher mesodermal defects (esophageal atresia) and lower mesodermal defects (anal atresia, genito-urinary defects)[11]. Genito-urinary malformations are well-described in triple X patients. Since many of the gastro-intestinal anomalies also affect the lower gastro-intestinal tract, perhaps a cloacal septation problem gives rise to the higher incidence of these types of malformations.

Guichet *et al.* reviewed prenatally and postnatally diagnosed 47,XXX karyotypes from 18 laboratories[35]. Mental retardation or congenital malformations were described in over one third of the 190 patients reported. In all cases weight-for-gestational-age at birth was under the 25$^{th}$ percentile. The authors did not report any gastro-intestinal anomalies. Our results are in agreement with these findings, with the exeption of the three cases found in our EA/TEF cohort.

The HUMARA assay demonstrated the maternal origin of the supernumerary X-chromosome in our triple X patients. RNA-FISH analysis and immunocytochemistry demonstrated inactivation of two out of three X-chromosomes with no skewed preference of a particular X-chromosome. Approximately 15% of the genes on the X-chromosome, located in the PAR regions, escape XCI[36]. The extent of this escape is tissue specific. Here, dosage compensation of gene expression on chromosome X is of interest[37-39]. Expression of genes on the inactive X can therefore result in low transcription levels[40]. Why and how certain genes escape XCI, especially in humans, is still unknown[41]. Female 'escapees' may have a dosage-sensitive function, which would explain the phenotype in patients with sex chromosome anomalies, such as Turner and Klinefelter syndrome. The observation that 47,XXX females also have decreased brain volume in the presence of normal pubertal maturation, suggests a possible direct dosage effect of X-chromosomal genes[27]. Similarly, other genes that escape XCI could perhaps cause the gastro-intestinal anomalies found in our cohort. One clear candidate in the PAR1 region is the *SHOX* gene. In two patients *SHOX* duplications were found. The *SHOX* gene encodes a cell-specific homeodomain protein that plays an important role during human embryonic bone and limb development[42,43]. A possible role for *SHOX* in limb/vertebrae anomalies as observed in the VACTERL association is not well established, however it is remarkable that EA/TEF patients with *SHOX* aberrations also have limb anomalies. All

pseudoautosomal genes, including *SHOX*, escape X-inactivation and are candidates for gene dosage effects when this region is deleted or duplicated. As a result of deletions and point mutations, *SHOX* haploinsufficiency is associated with idiopathic short stature (OMIM 300582)[44], and mesomelic short statured skeletal dysplasia (Leri-Will syndrome OMIM 127300)[45]. Homozygous loss of the *SHOX* gene has been correlated with Langertype mesomelic dysplasia (OMIM 249700)[45].

While haploinsufficiency of the *SHOX* gene has been related to different phenotypes, the association with *SHOX* duplications is less well-defined[19,46]. Thomas *et al.* could not define a distinct phenotype in four families with inherited *SHOX* duplications. There was female predominance (six out of eight cases) in these families, however our cases were two males[18]. It is difficult to define the clinical relevance of an inherited *SHOX* duplication and the possible role in the etiology of congenital anomalies when the parent is phenotypically normal[18,19]. Incomplete penetrance and variable expressivity have been described in microdeletion/duplication syndromes. Recently a two-hit model was proposed, in which the combinations of inherited and *de novo* copy number variations predisposed to neuropsychiatric phenotypes[47].



All five patients had duplicated loci of genes that escape XCI. The expression of these genes could possibly add up to the phenotype. As a consequence of the additional X-chromosome, triple X females express more genes that escape XCI. Furthermore, the expression levels of inactive X-chromosomes may vary between individuals and different tissues[48]. As previously described the phenotypic variability of Triple X syndrome ranges widely, from subclinical phenotypes to mental retardation and congenital malformations. Nevertheless, a increased gene dosage of X-linked genes may indeed have an impact on development, as suggested in a large study that characterized patients with mental retardation using an X-chromosome specific tiling array[49]. Also, mutations on chromosome X or elsewhere on the genome could contribute to this variability.

In conclusion, we describe five patients with sex chromosomal aberrations and EA/TEF. The incidence of this combination in our cohort is 11 times higher than expected compared to the general population. Screening of chromosome X with high-throughput sequencing, may reveal new X-linked syndromes related to gene dosage differences in congenital phenotypes such as EA/TEF. Improved copy number variations detection could reveal new duplication syndromes related to EA and other congenital anomalies.

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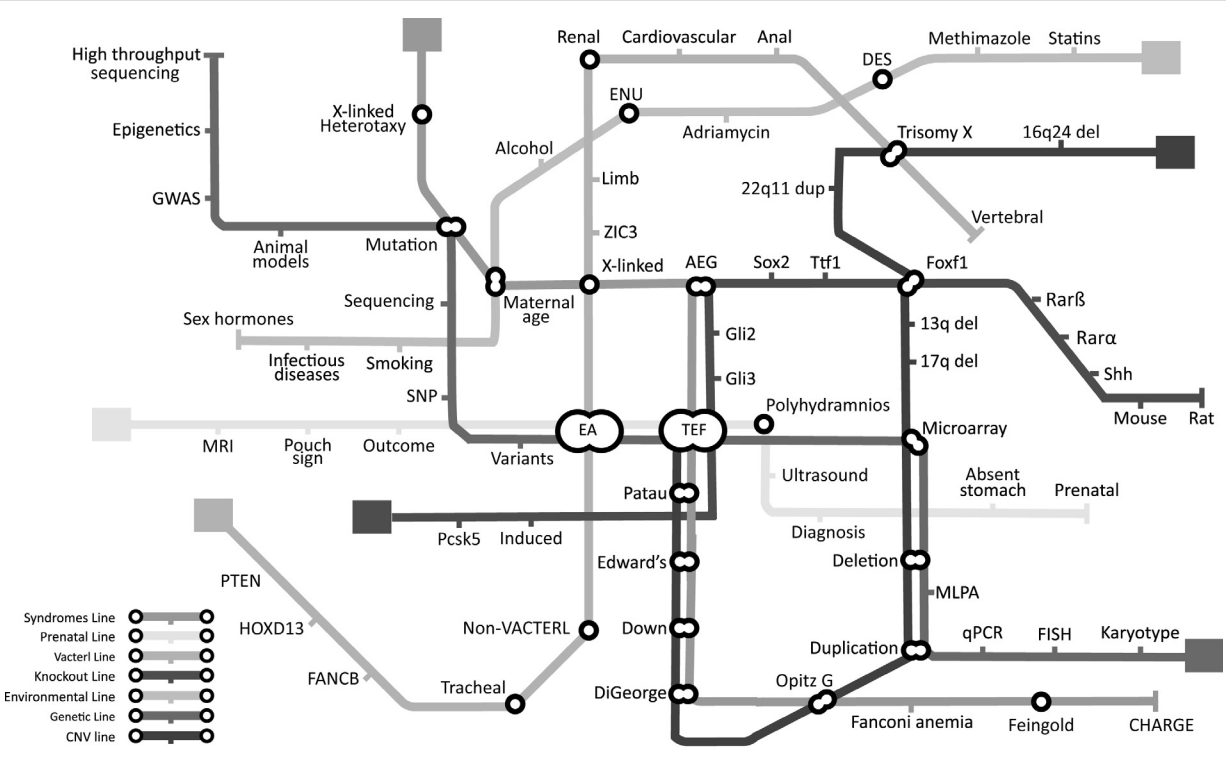
# CHAPTER 6

## 5q11.2 Deletion in a Patient with Tracheal Agenesis




Adapted from:

**EM de Jong**, H Douben, BH Eussen, JF Felix, MW Wessels, PJ Poddighe, PGJ Nikkels, RR de Krijger, D Tibboel, A de Klein. 5q11.2 deletion in a patient with Tracheal Agenesis. *Eur J Hum Gen* (provisionally accepted)

## Abstract

**Introduction:** Tracheal agenesis is a rare congenital anomaly of the respiratory tract. Many patients have associated anomalies, suggesting a syndromal phenotype.

**Methods:** In a cohort of twelve patients we aimed to detect copy number variations. In addition to routine cytogenetic analysis, we applied oligonucleotide Array CGH.

**Results:** Our patient cohort showed a variety of copy number variations of which many were parentally inherited variants. One patient had, in addition to an inherited 16p12.1 deletion, a 3.6 Mb deletion on chromosomal locus 5q11.2. This patient had a syndromic phenotype, including VACTERL associated anomalies, and other foregut-related anomalies, such as cartilage rings in the esophagus and an aberrant right bronchus.

**Conclusions:** No common deletions or duplications are found in our cohort, suggesting that tracheal agenesis is a genetically heterogeneous disorder.

## Introduction

**T**racheal **a**genesis (TA) is a rare congenital anomaly in which the trachea is fully or partially absent. Ventilatory support may be successful occasionally, but TA typically has fatal consequences. Efforts have been directed at clarifying the anatomy of TA and at improving treatment[1,2]. Tracheal agenesis may be part of a complex of malformations and it is often associated with cardiovascular anomalies[3,4]. The complex phenotypes show an overlap with the VACTERL association (**V**ertebral, **A**nal, **C**ardiovascular, **T**racheo-**E**sophageal, **R**enal and **L**imb anomalies)[5,6] or with an association including tracheal agenesis (TACRD; **TA**, **C**ardiac anomalies, **R**adial ray defects and **D**uodenal atresia)[6,7]. Tracheal agenesis can also be part of Fraser syndrome, an autosomal recessive malformation disorder characterized by cryptophthalmos, syndactyly, and abnormalities of the respiratory and urogenital tract, that is caused by mutations in the *FRAS1* and *FREM2* genes[8,9].

Little is known about the aetiology of TA, apart from the fact that environmental factors might play a role[3]. Animal models of different genetic defects, such as (conditional) inactivation of *Gli2*, *Gli3*, *Shh*, *Foxf1* and *β-Catenin*, show agenesis of the trachea or incorrect septation of the foregut[10-14]. To date, however, no mutations or chromosomal anomalies of any of the genes or pathways implicated in these animal models have been described in human patients with TA. In addition, there are no published reports on systematic screening of TA patients for copy number variations. To determine whether microdeletions/duplications could explain the TA phenotype, we conducted genome wide assays in twelve TA patients.



## Methods

### *Cases*

Ten cases were retrieved from the medical records of the Erasmus MC – Sophia Children's Hospital, Rotterdam, from 1988 onwards. Two cases had been seen and included at the department of Pathology, University Medical Centre Utrecht. The medical records and pathology records were reviewed. Fibroblast cell lines and paraffin blocks had been stored.

### *Cytogenetic analysis*

Karyotyping was performed according to standard analysis methods. DNA for genomic analysis was extracted from fibroblast cells by the Puregene DNA purification kit (Gentra Systems, USA*).* Regarding six patients, only Formalin Fixed Paraffin Embedded (FFPE) samples from thymus

tissue were available. DNA was extracted according to protocols provided by the supplier of the microarrays.

### *Array comparative genomic hybridization (Array CGH)*

Array CGH was performed using the 105K Oligonucleotide-based array CGH (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's protocol. For analysis and visualization the Feature Extraction software (version 9.1) and CGH analytics software (version 3.3.28) was used. Aberrations were detected with the ADM2 algorithm and filtering options of a minimum of 3 probes and abs(log2Ratio) >0.3. Aberration segments were manually reviewed.

### *FISH analysis*

To confirm the results, BAC clones were selected from the University of Santa Cruz (UCSC) genome browser and ordered from BACPAC Resources. After isolation of the Bac DNA the probes were labeled and used for Fluorescence In Situ Hybridization (FISH) on chromosome preparations from the patients and parents, according to standard protocols[15].

### *Real-time quantitative PCR*

Quantification was done by real-time quantitative PCR. Primer pairs were designed with Primer Express V2.0 (Applied Biosystems, Branchburg) and tested for validity. DNA was processed using the IQ SYBR green master mix (Biorad) and analysed on the AB7300 (Applied Biosystems, Branchburg).

### *Prioritization*

We used the Endeavour prioritization program[16] to prioritize the genes in the 5q11.2 chromosomal region. Reference genes were selected based on recently reviewed genes on foregut-related anomalies in human and murine models: *SHH, GLI2, GLI3, FOXF1, TTF1, NOG, FGF2, HOXA5, RARα, RARβ, BMP4, FOXF1, SOX2, FRAS1* and *FREM2*. In relation to cell cycle control and cell death processes, we performed an additional prioritization with selected reference genes: cell-division cycle 2-like genes, protein phosphatases, cyclin-dependent kinases, interleukins, apolipoprotein B, P53, TNF-genes, PDCD-genes, and CIDE genes. The program is based on the hypothesis that the novel candidate genes play roles similar to those of the genes known to be associated with the anomaly, or share biological processes with these genes.

## Results

Clinical data are summarized in Table 1. Conventional G-banding karyotyping was apparently normal in all patients. The quality of the array was poor in three from whom only FFPE material was available. The array CGH analysis revealed copy number variations in all patients. Most of these were known polymorphisms, described in the Toronto Database of Genomic variants (DGV), the Copy Number Variation project at the Children's Hospital of Philadelphia (CHOP), or seen in our in-house control cohort[17,18]. In two patients (Nos. 3 and 10) these were deletions of loci not known as polymorphic variants: a 57.9 kb deletion on chromosome 15q25.1, containing exons 12-21 of the *BLM* gene, and a 488 kb deletion on chromosome 16p12.1, respectively. The deletions were confirmed with FISH and real-time quantitative PCR. However, they were also found in their unaffected mothers, so we concluded that they were likely not causal.

We found an additional 3.6 Mb deletion on chromosomal locus 5q11.2 in patient 10 (Figure 1A), who presented with TA, anal atresia, persisting hemiazygos, cryptorchid testes, fork rib and mild dysmorphic features, including low set posterior rotated ears, mild hypertelorism and a unilateral simian crease. The pregnancy was achieved after *in vitro* fertilization with an unknown sperm donor, and had been complicated by polyhydramnios. In addition, absence of the stomach bubble was seen on ultrasound examination. Prenatal cytogenetic analysis was normal. As labour started prematurely, antenatal corticosteroids were given twice to induce lung development. The male baby was spontaneously delivered at 29 3/7 weeks. Body weight was 1090 g (30th centile), length 38 cm (50th centile), and occipitofrontal circumference 28 cm (40th centile). Cyanosis, severe respiratory distress and the absence of an audible cry were noted immediately after birth. Intubation failed after multiple attempts. Apgar scores were 3, 0 and 0 after 1, 5 and 10 minutes, respectively. Autopsy confirmed multiple congenital anomalies, including TA, which was classified as Floyd III[19]. In addition, cartilage rings in the esophagus and aberrant right bronchus were reported.

The deletion, ranging from 51.32–55.00 Mb (NCBI Build 36.1), was confirmed by FISH analysis (Figure 1B). Karyotyping and FISH analysis on lymphocytes of the mother were normal. Paternal material was not available because pregnancy has been achieved after *in vitro* fertilization; the sperm donor was unknown. Neither the DGV, and the CHOP nor our control cohort describes this large deletion. This chromosomal region contains twelve protein-encoding genes.

**Table 1: Patient characteristics and associated anomalies in TA cohort**

| Case no | Gender | Type TA | Gestation characteristics | Associated anomalies | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | V | A | C | TE | R | L | U | LA | D | Other |
| **1 *** | F | Floyd III | Polyhydramnios | - | - | - | - | - | - | - | + | - | - |
| **2** | M | Floyd III | Prolonged ROM<br>Maternal fever<br>Anhydramnios<br>Breech presentation | + | - | + | - | + | - | + | + | + | Gallbladder agenesis<br>Meckel's diverticulum |
| **3** | F | Floyd II with TEF | Polyhydramnios<br>Breech presentation<br>Cesarean section | - | - | + | + | - | - | - | + | + | Thymus atrophy<br>Extra spleen<br>Hypertelorism<br>Dysmorphic right ear<br>Single umbilical artery |
| **4** | M | Floyd III | Polyhydramnios | - | + | + | - | - | - | - | + | - | Hypertelorism<br>Immature brain |
| **5** | M | Floyd II | Prenatal diagnosis of multiple anomalies<br>IUGR; induction of labor | - | - | - | - | - | + | + | + | - | Low-set ears<br>Plagiocephaly<br>Ventriculomegaly and asymmetric skull<br>Clubfoot<br>Undescended testis |
| **6 *** | F | Floyd I with TEF | Polyhydramnios<br>Breech presentation<br>2nd of twins, other child healthy | - | - | - | + | - | + | - | - | - | Small left ear |
| **7 *** | M | Floyd I | IUGR | - | - | + | - | - | + | + | - | + | - |
| **8** | M | Floyd II with two TEF | - | - | + | + | + | - | - | + | - | - | - |
| **9** | M | Floyd I with TEF | Polyhydramnios<br>Vacuum extraction | - | + | + | + | + | - | - | + | - | Heterotaxy<br>Hypoplastic lungs |

| Case no | Gender | Type TA | Gestation characteristics | Associated anomalies | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | V | A | C | TE | R | L | U | LA | D | Other |
| **10** | M | Floyd III | Polyhydramnios<br>Induction of labour<br>IVF pregnancy | + | + | + | - | - | - | - | - | - | Aberrant right bronchus |
| **11** | F | Floyd I | Termination of pregnancy | - | - | - | - | - | + | - | - | - | Low-set ears<br>Hypertelorism<br>Hyperplastic lungs |
| **12** | M | Floyd II | Polyhydramnios | - | - | - | - | + | | - | - | - | Single umbilical artery |

*, Array inconclusive in Paraffin embedded tissue sample; F: female; M: male; TA: tracheal agenesis; TEF: tracheo-esophageal fistula; ROM: rupture of membranes; IUGR: Intra-Uterine Growth Retardation defined as weigh for gestational age <-2SD; TA: tracheal agenesis; V: vertebral and/or rib abnormalities; A: anal atresia; C: cardiovascular malformations; TE: esophageal atresia and/or tracheo-esophageal fistula; R: renal anomaly; L: limb abnormalities including radial ray defects; U: urogenital anomaly; LA: laryngeal defects; D: duodenal atresia; +: present; -: absent

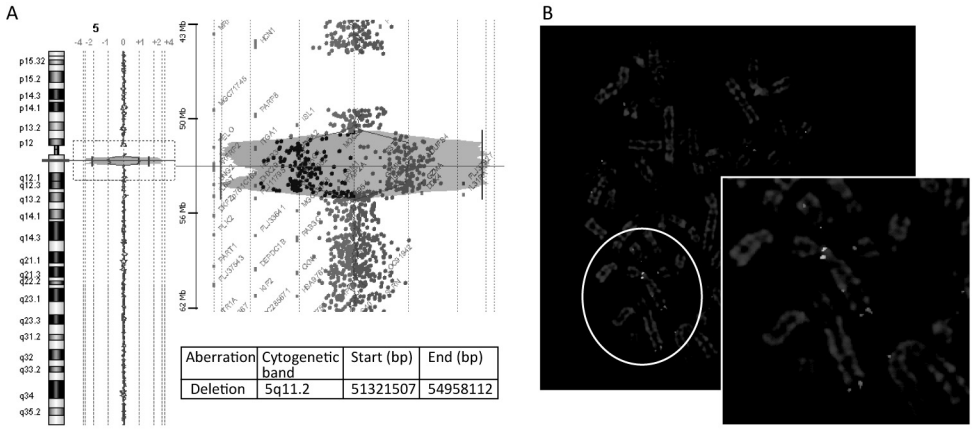



**Figure 1: Visualization and confirmation of deletion 5q11.2 by FISH**

A 5q11.2 deletion visualized with Agilent CGH analytics software (version 3.3.28); deviation from zero on a log2 scale, experiment performed in duplicate. The maximum deleted region ranged from 51,079,864-55,001,348 Mb (NCBI Build 36.1). The minimum deleted region ranged from 51,321,507-54,958,112 Mb.

B FISH to a metaphase spread, showing the absence of a green fluorescent signal (BAC RP11-160F8) at 5q11.2 on one chromosome 5. The red signal (BAC RP11-11K19; control probe) is present on both chromosomes on the 5q subtelomeric regions. *Color figures can be found in the appendix. See page 189*

## Discussion

As far as we know, one other patient with a deletion in the 5q11.2 region is described in the literature[20]. This patient showed Tetralogy of Fallot, a bifid uvula and significant developmental delay. The 5 Mb deletion found in this patient includes the 3.6 Mb region found in our patient. Another case of overlapping deletion was retrieved from a search in two public online databases (http://www.ecaruca.net/[21]; https://decipher.sanger.ac.uk/[22]). The Decipher database reports a patient with a large deletion del(5)(q11.2;q13.2), with a 2 Mb overlap with our patient. This patient had significant developmental delay and hypochondroplasia. To the best of our knowledge this deletion has not yet been described as a common variant. Due to lack of paternal material, we could not verify the inheritance of the deletion, but we expect this deletion to be *de novo*.

We used the Endeavour prioritization program[16] to prioritize the genes in the 5q11.2 chromosomal region whose haploinsufficiency may have influenced the congenital TA phenotype in our patient. Integrin alpha-1 (*ITGA1*) was ranked the highest priority, followed by Follistatin (*FST*), Endothelial cell-specific molecule-1 (*ESM1*) and Integrin alpha-2 (*ITGA2*). Integrin alpha-1 integrin is crucial in regulating mesenchymal stem cell proliferation and

cartilage production[23]. *FST* is an activin-binding protein. *FST*-deficient mice are growth retarded, have decreased intercostal muscles mass, skeletal defects, and abnormal tooth development[24]. The protein also modulates the actions of several members of the TGF-beta family, e.g. it inhibits *BMP-4*. Li *et al.* confirm that Bmp signaling is present in the anterior foregut, the site of origin of the tracheal primordium, and that targeted knockouts for *Bmp4* at this site have no trachea development[25]. Que *et al.* demonstrated that certain proteins that antagonize these BMPs, such as *Noggin* transcriptional processes, could explain the degree of tracheal development, and therefore could discriminate between the subtypes of TA[26].

The variable phenotypes in the patients described with 5q11.2 deletions can be potentially explained by different causes: environmental factors, incomplete penetrance, other genetic mutations elsewhere in the genome, compensation of other genes, and/or epigenetic factors. This patient in addition to the 5q11.2 deletion, also had an inherited 16p12.1 deletion, that could possibly contribute to the phenotype. This deletion was recently described by Girirajan *et al.*[27]. In addition to large *de novo* deletions, they identified a recurrent 520 kb microdeletion of 16p12.1 associated with development delay. They proposed a two-hit model: the inherited 16p12.1 deletion in addition to a large copy number variation indicates the variable expressivity of the developmental delay phenotype[27]. We were unable to ascertain the developmental status of the patient as he died. However as the mother is of normal intelligence and carries the 16p12.1 deletion, the patient could fit the two-hit model; the additional large copy number variation could indicate the variable expressivity in our patient.

The presence of inherited and polymorphic variations in our TA cohort suggests that TA is genetically heterogeneous. The rare 5q11 deletion found in one of our patients includes several candidate genes. The clinical significance of this large chromosomal aberration is hard to pinpoint, however, seeing the variability in the clinical presentation. We recommend standard detailed molecular cytogenetic analysis in patients with TA as a means to further unravel the genetic background of TA.

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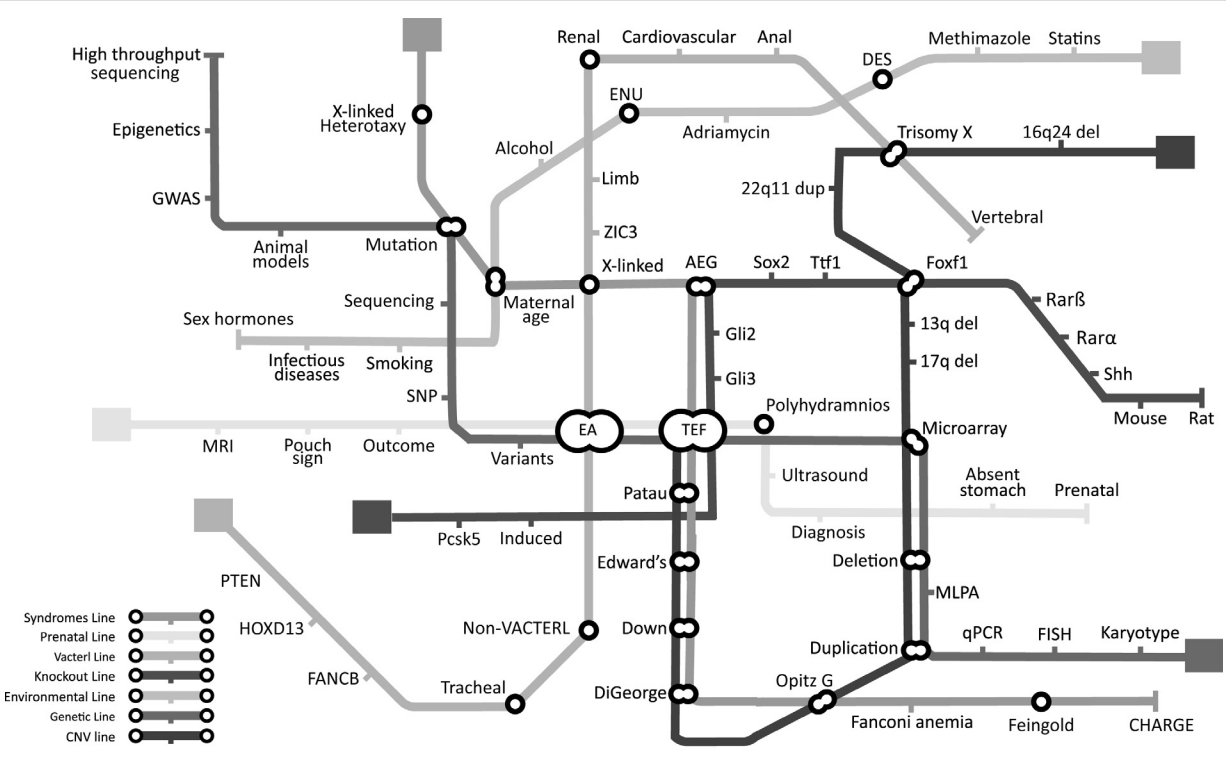
# CHAPTER 7

## Copy Number Variations in Patients with Esophageal Atresia and VACTERL Association




**Manuscript in preparation**

In collaboration with DA Scott, Baylor College of Medicine, Houston, USA

**EM de Jong** et al.

## Abstract

**Background: E**sophageal **a**tresia (EA) and/or **t**rachea-**e**sophageal **f**istula (TEF) are common life-threatening congenital anomalies. The cause is unknown in over 90% of cases. We investigated possible roles of copy number variations (CNVs) in de the development of EA/TEF and VACTERL-associated anomalies.

**Methods and Results:** A positional candidate approach based on chromosomal data generated with genome wide microarrays, yielded 56 inherited and six *de novo* CNVs, in a two center cohort of more than 200 patients. These variations were absent in published cohorts of normal individuals and in our in-house control cohort. In addition, we evaluated tissue of the esophageal pouch of 13 patients for somatic CNVs, but no change in CNV patterns nor loss of heterozygosity was found in a paired analysis with blood DNA of the corresponding patient.

**Conclusions:** Several loci, such as 4q35, 6q23.2, 7p22, 7p13, and 8q13.1 harbor new *de novo* copy number variations and some involve interesting disease related genes. Although identification of rare *de novo* CNVs would be considered strong evidence for causality, some of the inherited variants on chromosomal loci 5q11.2, 8p13.1, 10q25.3, 12p12.3, 19p13.3, and Xp22, were found in recurrently. This could suggest that the role of inherited variations may be underestimated and the majority of cases may result from a combination of inherited changes affecting important developmental pathways combined with environmental stressors.

## Introduction

**E**sophageal **a**tresia (EA) with or without **t**rachea-**e**sophageal **f**istula (TEF) is a common congenital malformation of the foregut, occurring in approximately 1 in 3,500 live births[1]. All foregut-related anomalies are thought to be complex diseases, with involvement of multiple genetic and environmental factors[2-4]. In 90% of cases the cause for EA/TEF is unknown, but a number of defined monogenic syndromes for which the underlying genes have been elucidated[5]. EA is a variable feature in e.g. Feingold syndrome (*MYCN*), CHARGE syndrome (*CHD7*), Opitz G syndrome (*MID1*), **A**nophthalmia-**E**sophageal-**G**enital (AEG) syndrome (*SOX2*), and in Fanconi anemia (multiple genes involved)[2]. Moreover, EA/TEF may be associated with cytogenetic abnormalities, including trisomies of chromosome 13, 18, 21, and X[6,7], deletions of chromosomal loci 22q11 (DiGeorge syndrome), 13q, 17q21.3-q24.2, and/or 16q24.1 in some cases[8-10].

EA/TEF is often associated with other structural birth defects; the VACTERL association (**V**ertebral, **A**nal, **C**ardiac, **T**racheo-**E**sophageal fistula, **R**enal, **L**imb; three or more anomalies present) is seen in approximately 10% of cases[11]. The high occurrence of associated anomalies seems to point at very many causes; an underlying genetic defect or possible environmental defects that involve more than one structure. Mutations in certain genes (*HOXD13, PCSK5*) or copy number variations (CNVs) (del 16q24.1) have been suggested to be the causative defect in a small number of VACTERL associated patients[10,12,13].

Genetic studies usually look for germline mutations as a cause of structural birth defects, although somatic mosaicism for copy number variations might also play a role. Non-affected individuals were assumed to have genetically identical normal cell types, but rearrangements of cells of the immune system display somatic mosaicism[14]. Recently it was found, however, that somatic mosaicism for stochastic CNVs occurs in a substantial percentage of cells[15]. It is now assumed that somatic CNVs must occur in somatic cells. Differences in CNVs between cell types of malformed tissue have not been investigated yet. In patients with congenital anomalies, such as EA/TEF, somatic mosaicism might explain the low levels of heritability.

Microarray studies have proved effectiveness in mapping and identifying genes that predispose to congenital anomalies in an unbiased fashion, such as non-isolated congenital diaphragmatic hernia[16], isolated tetralogy of Fallot[17] or neuro-developmental diseases[18,19]. Therefore, we applied a positional candidate approach to localize and identify genes that cause or predispose to the development of EA/TEF. We hypothesize that each of these CNVs harbors one of more EA/TEF-related genes.

## Methods

### *Cases*

Patients with EA/TEF and VACTERL associated anomalies were identified from the medical records of the Erasmus MC – Sophia Children's Hospital, Rotterdam, The Netherlands. In addition, patients referred to clinical geneticists at Baylor College of Medicine, Houston, USA, were included. This study was approved by the Medical Ethical Review Boards at both institutions. The medical records were reviewed for patient characteristics and genetic follow up. The VACTERL association was judged present if the records reported two or more anomalies of the VACTERL spectrum in addition to the EA/TEF.

### *Cytogenetic analysis and Multiplex ligation-dependent probe amplification (MLPA)*

Karyotyping was performed according to standard analysis methods. DNA for genomic analysis was extracted from blood and fibroblast cells by the Puregene DNA purification kit (Gentra Systems, USA). DNA was extracted from Formalin Fixed Paraffin Embedded (FFPE) samples (thymus tissue) as instructed by the supplier of the microarrays (Agilent®). All DNA was tested for subtelomeric aberrations with MLPA-analysis, using the P036E1 and P070A2 Salsa telomere kits (MRC Holland, Amsterdam, The Netherlands) as published previously[20].

### *Microarrays*

To identify CNVs, high-resolution analyses were performed using single-nucleotide polymorphism (SNP) microarrays (Illumina®, Affymetrix®) and oligonucleotide-based (105K, 244K, 1M) Array CGH (Agilent®). The 250K Nsp1 array (Affymetrix®) was used according to the manufacturer's instructions (http://www.affymetrix.com/). Imaging was done with the Affymetrix® GCS3000-G7 scanner. Genotype calls and probe intensities were extracted with the Affymetrix® GeneChip Command Console (AGCC) software version 1.1. Genotype- and CNV-analysis were performed using the BRLMM and CNAT 4.0.1 algorithms. CNVs in patient samples were visualized as log2 ratios detected by comparison to a common reference set of 40 CEU samples from the HapMap project (www.hapmap.org/downloads/raw_data/500K/). A second CNV analysis was done by comparison with an in-house run male control sample. The segment-reporting tool filter was set to a minimum of three aberrant markers per segment and a minimum segment size of 10Kb.

The Human 610-Quad BeadChip and the Human Cyto SNP-12 chip (Illumina®, San Diego, CA, USA) were used according to the manufacturer's protocol (http://www.illumina.com/). Both types of arrays were scanned with the Illumina Beadstation scanner and raw data

were uploaded in Beadstudio V2.0 genotyping software (Illumina®) for further analysis. CNVs were visualized with the log-normalized intensity ratio for each SNP in the patient sample and compared with a pooled reference set of 120 female CEU samples from the HapMap project. Heterozygous deletions or duplications were detected with a minimum of 5 consecutive SNPs.

In addition, paired analysis was performed for proximal pouch DNA and blood DNA samples. In the paired-sample analysis, the Log R Ratio for a sample is the log (base 2) ratio of the normalized R value for the SNP from the subject sample (blood) divided by the normalized R value from the reference sample (pouch).

For the SNP platforms, log2 ratios and the B-allele frequency (BAF) were visualized. with the Nexus® Copy Number software package version 4.0 (BioDiscovery Inc., El Segundo, CA, USA).

Array CGH was performed using the 244K, the 105K and the 1M oligonucleotide-based array CGH platforms (Agilent® Technologies, Santa Clara, CA, USA), according to the manufacturers protocol. Genomic DNA from the patient and reference DNA (Promega Corporation, Madison, WI), pooled normal male and female, was labeled with Cy5 and Cy3 respectively. The 244K and 1M arrays were done with sex matched controls of unaffected, unrelated individuals. Correct labelling was checked using the NanoDrop Spectrophotometer ND1000 (NanoDrop Technologies). For the FFPE samples, patient and reference DNA were labeled using the Oligo Array CGH Labelling kit for FFPE samples (Agilent® Technologies). For analysis and visualization the Feature Extraction software (version 9.1) and CGH analytics software (version 3.3.28) was used. Aberrations were detected with ADM2 algorithm and filtering options of a minimum of 3 probes (1M and 105K) or 2 probes (244K), and abs(log2Ratio) > 0.3. Aberration segments were manually reviewed.



### *Validation of microarray results*

All results were checked against our in-house control database of common CNVs found in healthy controls, the Toronto Database of Genomic Variants (DGV) and the Copy Number Variation project at the Children's Hospital of Philadelphia (CHOP)[21,22].

To confirm the results, quantification of the DNA was done by Real Time RT-PCR and/or Fluorescence *In Situ* Hybridization (FISH) on chromosome preparations from the patients and parents. Primer pairs were designed with Primer Express V2.0 (Applied Biosystems, Branchburg), the OLIGO 6.0 Software, or Primer3Plus and tested for validity. DNA was processed using the IQ SYBR green master mix (Biorad) and analyzed on the AB7300 (Applied Biosystems, Branchburg), according to standard protocols. Each sample, including the no template control (NTC) and control DNA, was run in triplicate. Exon 4 of the KIAA1279-gene

served as a control locus. Values of < 0.7 and > 1.3 served as boundaries for deletions and duplications respectively.

For FISH validation, BAC clones were selected from the University of Santa Cruz (UCSC) genome browser and ordered from BACPAC Resources. After isolation of the BAC DNA the probes were labeled and used for FISH, according to standard protocols[23].

*Prioritization*

We used the Endeavour prioritization program[24] to prioritize the genes found in all the *de novo* and inherited aberrations. Reference genes were selected based on recently reviewed genes on foregut-related anomalies in human and murine models: *SHH, GLI2, GLI3, FOXF1, TTF1, NOG, FGF2, HOXA5, RARα, RARβ, BMP4, FOXF1, SOX2*. The program is based on the hypothesis that the novel candidate genes play roles similar to those of the genes known to be associated with the anomaly, or share biological processes with these genes.

## Results

The study cohort consisted of over 200 patients. Patients with defined syndromes, such as trisomies or proven mutations in disease causing genes in Feingold and AEG syndrome, had been excluded.

Over 60% of patients showed other major congenital anomalies, predominantly VACTERL associated anomalies. A classification was made into: isolated cases, EA/TEF and heart anomalies, EA/TEF and anal anomalies, and VACTERL associated patients. These subgroups were not mutually exclusive, as a large part of the VACTERL patients were also included in the heart and/or anal subgroup.

The results of the different platforms are summarized in Table 1. Genomic changes were considered rare variants if similar changes were not found in databases of unaffected, unrelated individuals (http://projects.tcag.ca/variation/[21]; http://cnv.chop.edu/[22]) or in an in-house database of unaffected control individuals. All rare variants found were confirmed by real-time quantitative PCR and/or FISH. An example of the investigation of a variant is given in Figure 1.

We compared affected tissue (proximal pouch) and peripheral blood DNA for somatic changes. Of 13 patients, DNA from proximal pouch tissue could be extracted. In four patients a paired analysis could be performed with the blood DNA of the corresponding patient on the Illumina SNP platform. This did not reveal change in CNV patterns nor loss of heterozygosity.

The other 9 tissue/blood DNA pairs were manually compared for copy number changes. Over 90% of the changes were seen in both tissues samples; they were mainly polymorphic or inherited variants and did not include possible candidate genes.

**Table 1: Copy number variations in patients with EA/TEF and VACTERL association**

| Type | Chr. | Band | Mb | Size | Genes affected | Confirmation |
|---|---|---|---|---|---|---|
| Del | 1 | q24.2 | chr1:166,707,913-166,964,241 | 256 kb | *XCL2, XCL1, DPT* | Inherited - Mat |
| Del | 1 | q25.2 | chr1:177,954,393-178,059,341 | 104 Kb | *FAM163A* | Inherited - Pat |
| Dup | 1 | q31.1 | chr2:176,828,859-176,965,152 | 136 Kb | *MTX2* | Inherited - Mat |
| Del | 2 | p22.1 | chr2:39,835,726-39,898,547 | 63 Kb | *THUMPD2* | Inherited - Mat |
| Del | 2 | p15 | chr2:62,058,607-62,081,510 | 23 Kb | *COMMD1* | Inherited - Pat |
| Del | 2 | p14 | chr2:66,539,109-66,625,441 | 86 Kb | *MEIS1* | Inherited - Mat |
| Dup | 2 | q36.3 | Chr2: 228,069,192- 228,082,423 | 13.2 Kb | *TM4SF20* | Inherited - Pat |
| Del | 3 | q13.12 | chr3:108,892,833-109,053,887 | 161 Kb | *BBC, LOC151658* | Inherited - Pat |
| Del | 4 | p16.1 | chr4:7,761,879-7,810,539 | 49 Kb | *SORCS2, AFAP* | Inherited - Pat |
| Dup | 4 | q12 | chr4:54,846,096-54,874,109 | 28 Kb | *PDGFRA* | Inherited - Mat |
| Del | 4 | q35.2 | chr4:189,262,840-189,279,737 | 16.9 Kb | *TRIML2* | *De Novo* |
| Del | 5 | p13.1 | chr5:40,814,789-40,821,601 | 6.8 Kb | *PRKAA1* | Inherited - Pat |
| Del | 5 | q11.2 | chr5:54,127,000-54,369,473 | 242 Kb | *ESM1, GZMK* | Inherited - Pat |
| Del | 5 | q11.2 | chr5:54,133,714-54,401,751 | 268 Kb | *ESM1, GZMK* | Inherited - Pat |
| Del | 6 | p22.1 | chr6:26,580,422-26,611,236 | 31 Kb | *BTN1A1* | Inherited - Mat |
| Dup | 6 | q14.1 | chr6:76,268,597-76,491,113 | 223 Kb | *SENP6* | Inherited - Pat |
| Dup | 6 | q16.3 | chr6:100,426,259-100,576,358 | 150 Kb | *MCHR2* | Inherited - Pat |
| Del | 6 | q23.2 | chr6:134,772,684-135,062,237 | 289 Kb | *No* | *De Novo* |
| Del | 6 | q24.1 | chr6:142,513,441-142,660,062 | 147 Kb | *VTA1* | Inherited - Mat |
| Del | 7 | p22 | chr7:6,090,284-6,106,523 | 16.2 Kb | *Distal to USP42* | *De Novo* |
| Del | 7 | p13 | chr7:43,592,420-43,597,873 | 5.5 Kb | *STK17A* | Homozygous Del *De Novo* |
| Del | 7 | p12.3 | chr7:48,556,504-48,562,351 | 5.8 Kb | *ABCA13* | Inherited - Mat |
| Del | 7 | p12.3 | chr7:48,556,504-48,562,351 | 5.8 Kb | *ABCA13* | Inherited - Mat |
| Dup | 7 | q11.23 | chr7:72,338,350-74,208,716 | 1.87 Mb | *Multiple* | Inherited - Pat |
| Del | 7 | q22.1 | chr7:101,907,906-102,123,873 | 216 Kb | *POLR2J2, POLR2J3, RASA4, SPDYE2, UPLP* | Inherited - Pat |
| Del | 7 | q34 | chr7:142,535,765-142,600,931 | 65 Kb | *PIP, TAS2R39* | Inherited - Mat |
| Dup | 8 | q13.1 | chr8:67,118,081-67,126,592 | 8.5Kb | *DNAJC5B* | Inherited – Pat * |
| Dup | 8 | q13.1 | chr8:67,118,081-67,143,367 | 25 Kb | *DNAJC5B* | Inherited – Pat * |
| Dup | 8 | q13.1 | chr8:67,118,081-67,143,367 | 25 Kb | *DNAJC5B* | *De Novo* |
| Del | 8 | q13.3 | chr8:71,779,310-71,789,822 | 10.5 Kb | *XKR9* | Inherited - Mat |

| Type | Chr. | Band | Mb | Size | Genes affected | Confirmation |
|---|---|---|---|---|---|---|
| Del | 8 | q13.3 | chr8:71,779,310-71,789,822 | 10.5 Kb | *XKR9* | Inherited - Pat |
| Del | 8 | q21.3 | chr8:91,676,176-91,824,081 | 147 Kb | *TMEM64* | Inherited - Pat |
| Dup | 10 | p11.22 | chr10:33,133,081-33,321,872 | 189 Kb | *ITGB1* | Inherited - Pat |
| Del | 10 | p11.21 | chr10:35,916,849-35,950,279 | 33 Kb | *GJD4* | Inherited - Pat |
| Dup | 10 | q25.3 | chr10:116,250,269-116,546,953 | 296 Kb | *ABLIM1* | Inherited - Mat |
| Dup | 10 | q25.3 | chr10:116,261,258-116,515,586 | 254 Kb | *ABLIM1* | Inherited - Mat |
| Del | 11 | p15.4 | chr11:8,915,512-8,919,362 | 3.9 Kb | *ASCL3* | Inherited - Mat |
| Del | 11 | q14.2-14.3 | chr11:86,775,384-91,610,110 | 4.8 Mb | *multiple* | Inherited - Pat |
| Dup | 12 | p13.31 | chr12:7,894,681-8,009,303 | 115 Kb | *SLC2A14, NANOGP1, SLC2A3* | Inherited - Mat |
| Del | 12 | p12.3 | chr12:16,035,488-16,170,844 | 135 Kb | *DERA* | Inherited - Mat |
| Del | 12 | p12.3 | chr12:16,047,790-16,164,597 | 117 Kb | *DERA* | Inherited - Mat |
| Del | 12 | q12 | chr12:39,429,168-39,438,782 | 9.6 Kb | *CNTN1* | Inherited - Pat |
| Dup | 12 | q13.2 | chr12:55,025,975-55,122,663 | 97 Kb | *STAT2, APOF, TIMELESS* | Inherited - Mat |
| Dup | 12 | q24.12 | chr12:110,668,304-110,802,629 | 134 Kb | *ACAD10, ALDH2, PNAS-1, MAPKAPK5* | Inherited - Mat |
| Del | 13 | q12.3 | chr13:28,612,594-28,703,794 | 91 Kb | *KIAA0774* | Inherited - Pat |
| Del | 13 | q21.33 | chr13:69,261,108-69,678,603 | 417 Kb | *KLHL1 (B)* | Inherited - Pat |
| Del | 15 | q13.3 | chr15:30,202,944-30,653,250 | 450 Kb | *CHRNA7, FAM7A* | Inherited - Pat |
| Dup | 15 | q21.3 | chr15:53,194,652-53,737,840 | 543 Kb | *CCPG1, DYX1C1, PIGB, PRTG, PYGO1, RAB27A, RSL24D1* | Inherited - Mat |
| Del | 15 | q26.1 | chr15:87,149,810-87,164,592 | 14.8 Kb | *ACAN* | Inherited - Mat |
| Dup | 16 | q24.3 | chr16:8,671,293-9,200,858 | 530 Kb | *ABAT, TMEM186, PMM2, CARHSP1, USP7* | Inherited - Mat |
| Del | 18 | p11.31 | chr18:6,092,791-6,102,462 | 9.7 Kb | *L3MBTL4* | Inherited - Mat |
| Dup | 18 | p11.31 | chr18:5,460,006-5,550,340 | 90 Kb | *EPB41L3* | Inherited - Mat |
| Del | 19 | p13.3 | chr19:2887367-2915807 | 28 Kb | *ZNF77* | Inherited - Pat |
| Del | 19 | p13.3 | chr19:595,208-602,228 | 7 Kb | *RNF126, FLJ45684* | Both Parents Del |
| Del | 19 | p13.3 | chr19:4,350,702-4,354,182 | 3.5 Kb | *SH3Gl1, CHAF1A* | Inherited - Mat |
| Del | 19 | p13.3 | chr19:4,350,702-4,354,182 | 3.5 Kb | *SH3Gl1, CHAF1A* | Inherited - Mat |
| Del | 20 | 13.13 | chr20:48,242,315-48,242,775 | 0.5 Kb | *CEBPB* | Inherited - Mat |
| Dup | 22 | 11.2 | chr22:17,147,764-18,648,997 | 1.5 Mb | *multiple incl. TBX1* | Inherited - Mat |
| Del | 22 | 13.31 | chr22:46,188,483-46,861,153 | 672 Kb | *none* | Inherited - Mat + unaffected twin |
| Dup | 22 | q13.33 | chr22:48958906-48966272 | 7.4 Kb | *PANX2* | Inherited - Mat |
| Del | X | p22.2 | chrX:13,878,418-14,021,051 | 142 Kb | *GEMIN8* | Inherited - Mat |
| Dup | X | P22.33 | chrX:501,091-693,629 | 102 Kb | *SHOX* | Inherited - Mat |
| Del | X | q13.1 | chrX:69,378,166-69,383,712 | 5.5 Kb | *Distal to DGAT2L3* | Inherited - Mat |
| Dup | X | 47,XXX | chrX:1-154,913,754 | 155 Mb | *whole chromosome* | *De Novo* |

| Type | Chr. | Band | Mb | Size | Genes affected | Confirmation |
|---|---|---|---|---|---|---|
| Dup | X | 47,XXX | chrX:1-154,913,754 | 155 Mb | *whole chromosome* | *De Novo* |
| Dup | X | 47,XXX | chrX:1-154,913,754 | 155 Mb | *whole chromosome* | *De Novo* |
| Dup | X/Y | p22.33 | chrX:248,968-1,229,976 | 981 Kb | *SHOX, PPP2R3B* | Inherited - Pat |
| Dup | Y | q11.2 | chrY:14,225,506-14,603,722 | 378 Kb | *TMSB4Y* | Inherited - Pat |

Genomic changes found in a cohort of >200 patients with EA/TEF and VACTERL association. Confirmation of the rare variants and their inheritance pattern was done by real time quantitative PCR if similar changes were absent in published cohorts of normal individuals and in our in-house control cohort. The recurrent aberrations are presented in the darkgrey fields. Del, deletion; dup, duplication; Pat, paternal; Mat, maternal; *, three patients with same deletion. Base pair positions according to Build 36.1/hg18.

Recurrent aberrations were found for several genes including *SHOX*, *TBX1*, *TRIML2*, *ESM1*, *ABLIM*, *ITGB1*, and *MEIS1*. With the Endeavour prioritization tool genes were prioritized according to their function processes and relations to genes involved in foregut development. The top 10 genes according to this analysis are: *CEBPB, SHOX, PDGFRA, ITGB1, MEIS1, TBX1, SH3GL1, STK17A, CHRNA7,* and *ALDH2*.

## Discussion

In many genetic disorders candidate genes can be determined by a linkage-analysis approach. In most EA/TEF cases, however, there is no familial recurrence. In congenital anomalies with mostly a *de novo* occurrence, genomic rearrangements may explain the human phenotypic variation and susceptibility to disease[17-19]. We therefore used high-resolution oligonucleotide and SNP microarrays to identify possible CNVs. This approach yielded both *de novo* and inherited CNVs, widely distributed over the genome. The identified regions included some interesting genes such as *SHOX, TBX1, TRIML2, ESM1, ABLIM, ITGB1*, and *MEIS1*. However, large recurrent variants were not found, so a positional candidate approach is less applicable. Studies describing somatic mosaicism for CNVs in different cell types are rare[15]. In our cohort we were able to check differences in CNVs between the proximal pouch and blood cells, but manual comparison and a paired analysis did not detect somatic differences. Comparison of the recorded CNV data with known EA/TEF loci from animal models or recorded chromosomal anomalies yielded no overlapping CNVs. In none of the regions describing a new variant imprinted genes were discovered.

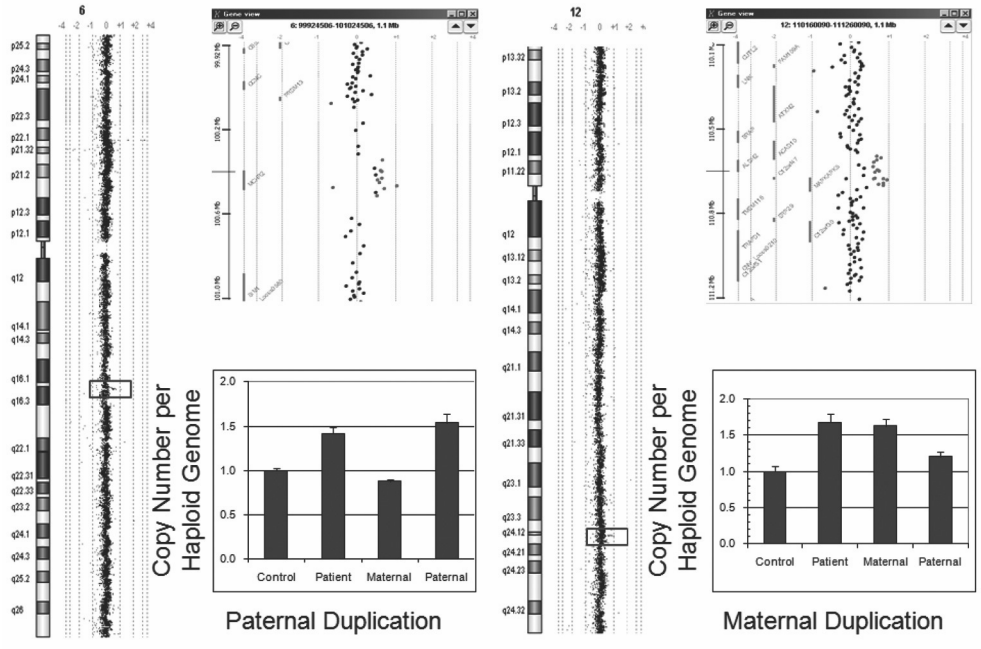



**Figure 1: Identifying genomic alterations in patients with EA/TEF and VACTERL association.**
Examples of array comparative genome hybridization data from 244K Agilent arrays depict duplication events on chromosome 6 and chromosome 12. Quantitative real-time PCR is used to confirm the changes and to determine the inheritance pattern within families. In this case the duplication on chromosome 6 was inherited from the patient's father and the duplication on chromosome 12 from the patients mother. Color figures can be found in the appendix. See page 189

### *De novo variants*

Several loci such as 4q35, 6q23.2, 7p22, 7p13, and 8q13.1 harbor new *de novo* CNVs and some involve interesting disease related genes. A possible *de novo* deletion of the tripartite motif family-like 2 gene (*TRIML2*) was found. *TRIML2* is expressed in whole brain and in lymphoblast B-cells, but no studies in relation to pathogenicity and disease have been published until now. A 289 Kb deletion of 6q23.2 was described in a patient who showed dysmorphic features and a ventricular septal defect. However, this locus did not involve any genes. A 5.9 Mb deletion of 7pter was found in one patient. Deletions of 7p are cytogenetically heterogeneous and result in a wide spectrum of congenital abnormalities, minor facial and hand anomalies, and often including craniosynostosis. Terminal deletions of 7pter are rare, but EA/TEF was not yet described for this deletion[25,26]. None of these variants were described in EA/TEF patients so far. Additionally, we reported three females with the Triple X syndrome and two patients with

inherited duplications of *SHOX* within the X/Yp PAR1 region. Gastro-intestinal anomalies have not been described in patients with *SHOX* duplications so far, and were reported in only a small number of individuals with triple X syndrome[27,28].

### *Inherited variants*

Non-pathogenic and/or inherited CNVs were predominant in our cohort, and many have already been described in unaffected individuals[21,22]. Inheritance from a phenotypically normal parent favors the interpretation of a benign variant[29,30]. Still, the phenotype of a genomic disorder can be variable due to variable gene expressivity, incomplete penetrance, skewed X-inactivation and/or mutations elsewhere in the genome. Some genomic alteration syndromes, such as microdeletions of 3q29 and microduplications of 7q11.23 and 22q11, seem to have a lower penetrance[31-33]. Incomplete penetrance was noted in our cohort, too; the patient with an inherited 22q11 microduplication syndrome had a VACTERL phenotype and his mother only had mild dysmorphic features[33,34]. Additionally, we reported two males with inherited duplications of the The Short stature HOmeoboX-containing gene (*SHOX*) [MIM312865]. This gene is located within the pseudoautosomal region (PAR1) of X(pter) and Y(p) and was also prioritized. *SHOX* haploinsufficiency, as a result of deletions and point mutations, is associated with multiple growth-related phenotypes[35-37]. However, with limited *SHOX* duplications published, no distinct phenotype can be established[38,39]. The recurrent aberrations involving ABLIM1 [MIM:602330] and ESM1 [MIM:601521] are also of interest. ABLIM1, an actin-binding LIM domain protein, functions as protein-binding interfaces, mediating specific protein-protein interactions[40] and plays important roles in regulating developmental pathways[41]. ESM1, endothelial cell-specific molecule 1, is mainly expressed in the endothelial cells in human lung and kidney tissues. The expression is regulated by cytokines suggesting that it may play a role in endothelium-dependent pathological disorders, especially colorectal cancer[42,43].

In a recent study, Girirajan *et al.* described a recurrent 520 kb large 16p21.1 microdeletion in a cohort of patients with developmental delay. Comparison with other large cohorts of unaffected controls and affected patients provided evidence of a two-hit model in patients with inherited 16p12.1 deletions. These deletions are often inherited from an unaffected parent, and occur in association with a large copy number variation that could explain the variability in these and other microdeletion syndromes[44]. The two-'hit' model represents the assumption that two modifiers add up to each other's effects or that it is part of an epistatic process, which in this case created a severe phenotype of developmental delay. In addition the CNVs themselves could explain reduced penetrance of disease-causing

mutations; there is evidence that a dominant loss-of-function mutation might be rescued by a gain in copy number resulting in increased gene expression[45]. However, our data do not support this theory and therefore the other causal theory of incomplete penetrance for CNVs in EA/TEF patients still stands.

After prioritization some interesting genes were discovered. In one patient, the 22q11 microduplication syndrome, inherited from his mother, was found. This included the *TBX1* gene, a dosage sensitive gene, responsible for the phenotype in DiGeorge syndrome [MIM 188400][46]. The phenotype driven detection approach clearly explains the heterogeneous phenotype of patients with 22q11 microduplications[47]. Until now, 22q11 microduplication was not described in patients with EA/TEF.

Data of cohorts of EA/TEF patients screened for CNVs with single-nucleotide polymorphism microarrays and oligonucleotide-based Array CGH will be available soon. These can be used in genome wide association studies aiming to identify susceptibility loci for EA/TEF. This approach already proved successful in other congenital anomalies, such as non-syndromic cleft lip/palate and replicated in independent samples[48,49]. GWAS studies require large sample sizes to obtain sufficient statistical power. Mangold *et al.* found relative risks of 1.8 (95% CI interval) for two loci in 401 individuals with cleft lip with or without cleft palate[49]. These data provide evidence for the success rate found in other congenital anomalies, based upon the genetic heterogeneity of the disorder.

Microarrays are expected to fully replace karyotyping in diagnostic screening for underlying causes in congenital anomalies. Translocations will still be missed, but microarrays are already routinely used in patients with mental retardation[50]. The data presented in this study provide limited evidence for the causal effects of CNVs in EA/TEF. We found only few *de novo* changes. Platforms with higher resolutions, such as the Affymetrix 6.0 GeneChip SNP arrays or the Illumina CytoChips, could allow for more detailed analysis. On the other hand, such approaches may lead to data interpretation problems such as high false positive rates and the discovery of smaller CNVs of unknown clinical significance[51]. Studies of healthy control populations are necessary to understand the implications of specific CNVs in congenital disorders, such as EA/TEF and foregut-related anomalies. However, with the advances in high-resolution techniques, including whole genome sequencing, the search for underlying genetic defects will intensify.

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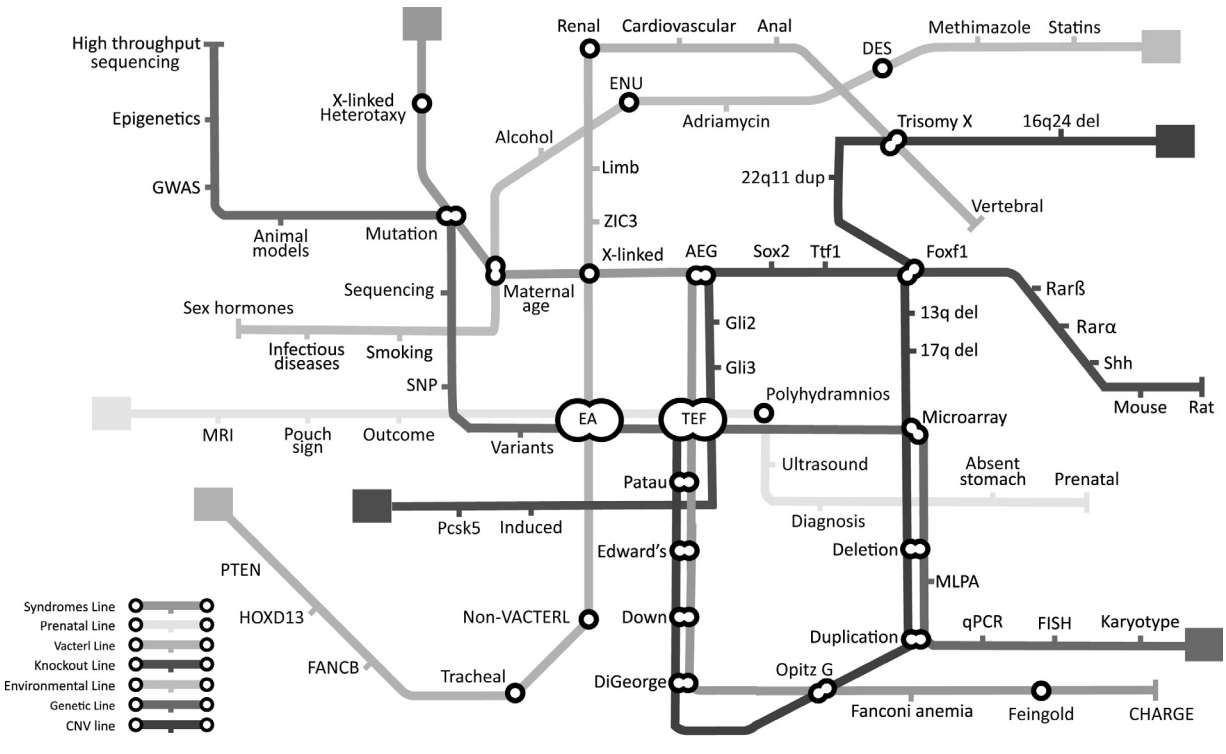
# CHAPTER 8

## Polyalanine Expansion in the ZIC3 Gene Leading to X-linked Heterotaxy with VACTERL Association: A New Polyalanine Disorder?




**Adapted from:**

Wessels MW, Kuchinka B, Heydanus R, Smit BJ, Dooijes D, de Krijger RR, Lequin M, **de Jong EM**, Husen M, Willems PJ, Casey B. 2010. Polyalanine expansion in the ZIC3 gene leading to X-linked heterotaxy with VACTERL association, a new polyalanine disorder ? J Med Genet *In press*.

## Abstract

**Case report:** We describe a newborn male with features of the VACTERL association, including anal atresia, laryngeal and esophageal atresia with tracheo-esophageal fistula, dextroposition of the heart with persistent left superior vena cava, and unilateral multicystic kidney.

**Methods and Results:** As the clinical picture of the VACTERL association overlaps with X-linked heterotaxy caused by *ZIC3* mutations, we sequenced the *ZIC3* coding region, and found a 6-nucleotide insertion that is predicted to expand the amino-terminal polyalanine repeat from ten to twelve polyalanines. This novel mutation was not present in the mother, nor in 336 chromosomes from 192 ethnically-matched controls.

**Conclusion:** We hypothesize that this novel and *de novo* polyalanine expansion in the *ZIC3* gene contributes to the VACTERL association in this patient.

## Introduction

Mutations in *ZIC3*, a zinc finger transcription factor gene located at Xq26, typically result in a spectrum of left-right asymmetry defects, including complex cardiac anomalies, altered lung lobation, splenic and hepatobiliary abnormalities, and gut malposition. Also renal, anal and lumbosacral anomalies are common. The combination of *ZIC3*-associated anomalies is referred to as X-linked heterotaxy (HTX1, MIM 306955)[1-5]. However, also isolated congenital heart disease can be due to mutations in *ZIC3*[4]. Though usually unaffected, some female heterozygotes manifest abnormalities whose spectrum and severity is indistinguishable from affected males.

The VACTERL association, which comprises **v**ertebral anomalies (V), anal **a**tresia (A), **c**ardiovascular malformations (C), **t**racheo-esophageal fistula and/or **e**sophageal atresia (TE), **r**enal malformations (R) and **l**imb defects (L) is a non-random association of defects with an unknown etiology in the majority of patients[6,7]. In several recent reviews the syndromes resembling VACTERL association have been discussed: these include Feingold syndrome (*NMYC* gene), Fanconi syndrome (*FANC* genes), CHARGE syndrome (*CHD7* gene), Pallister-Hall syndrome (*GLI3* gene), Anophthalmia-Esophageal-Genital syndrome (*SOX2* gene), Opitz G/BBB syndrome (*MID1* gene), Townes-Brocks syndrome *(SALL1* gene*)* and Fryns syndrome (disease gene not identified yet)[8,9,10]. VACTERL association phenotypically also overlaps with *ZIC3*-associated X-linked heterotaxy: anal atresia and cardiac defects are common in both disorders, and typical VACTERL features such as tracheo-esophageal fistula, renal and vertebral anomalies have occasionally also been reported in *ZIC3*-associated heterotaxy (Table 1)[5].

In the case presented here, we describe a novel *ZIC3* mutation (elongation of the amino-terminal alanine repeat) in a patient with overlapping features between VACTERL association and X-linked heterotaxy.

## Case report

A healthy Caucasian gravida 2 para 1 was referred for ultrasound examination at 31 weeks of gestation because of suspected polyhydramnios. Apart from polyhydramnios advanced ultrasonography revealed absent stomach filling, suggesting esophageal atresia, and a right multicystic kidney. Amniocentesis showed a normal male karyotype, and FISH analysis to exclude a 22q11.2 deletion was normal. In her previous pregnancy the mother delivered a healthy son of 4,500 grams at 41 weeks of gestation. There were no congenital anomalies in the family of both nonconsanguineous parents.

**Table 1:** ***ZIC3*** **mutations in patients with features overlapping X-linked heterotaxy and VACTERL**

| *ZIC3* mutation | M/F | Heterotaxy | Cardiovascular | Vertebral | Laryngeal Oesophageal | Anal | Renal | Other | Ref |
|---|---|---|---|---|---|---|---|---|---|
| p.Arg183Gln | M | Asplenia<br>Altered abdominal situs | TGA<br>AVSD<br>Pulmonary atresia | | | Posterior-placed anus | | Arhinencephaly | [1,3] |
| p.Arg183Gln | M | Dextrocardia<br>Altered abdominal situs | TGA<br>Pulmonary valve stenosis<br>Mitral valve atresia | Sacral agenesis | | Rectal stenosis | | Biliary atresia<br>Bilateral clubfeet | [1,3] |
| c.1507insTT | F | Situs inversus | | | | | Double ureter | | [3] |
| c.1507insTT | M | Situs ambiguus | Not specified | | | Anal malformation | Horseshoe kidney | Adrenal aplasia | [3] |
| c.1507insTT | M | Situs ambiguus | Not specified | | | | Renal hypoplasia<br>Ureteral stenosis | | [3] |
| c.1507insTT | F(2) | Situs inversus | | | | Anal malformation | | | [3] |
| p.Gln294X | M(2) | Situs ambiguus | Not specified | | | Anal malformation | | | [3] |
| p.Cys270X | M | Situs ambiguus | Not specified | Sacral agenesis | | | | | [3] |
| p.Cys270X | M | Situs ambiguus | Not specified | | | Anal malformation | | | [3] |
| p.Cys253Ser | NR | NR | NR | Lumbosacral spine abnormalities | | | Horseshoe kidney | Omphalocele<br>Cystic hygroma | [5] |
| p.Gln249X | M | Altered abdominal situs<br>Asplenia | AVSD<br>DORV, TGA<br>Pulmonary stenosis<br>TAPVR | | | | Horseshoe kidney | | [5] |
| p.Gln249X | M | Dextrocardia<br>Asplenia | AVSD<br>Pulmonary atresia<br>HLHS<br>Bilateral superior vena cava | Vertebral defect | TF | Anal atresia | Renal dysplasia | Radial dysplasia | [5] |

| *ZIC3* mutation | M/F | Heterotaxy | Cardiovascular | Vertebral | Laryngeal Oesophageal | Anal | Renal | Other | Ref |
|---|---|---|---|---|---|---|---|---|---|
| p.Ser43X | M | Normal situs | DORV, TGA<br>ASD, VSD<br>Pulmonary stenosis<br>Interrupted inferior vena cava | Fused lumbar vertebrae | | Anal atresia | | Bilateral clubfeet<br>Posterior embryotoxon<br>Extrahepatic biliary atresia | 5 |
| Deletion Xq26 | M | Intestinal malrotation<br>Midline stomach, liver<br>Asplenia | AVSD<br>Left superior vena cava | | | Anal atresia | | | 2 |
| Deletion Xq26 | M | Bilateral trilobated lungs<br>Asplenia<br>Right stomach | AVSD, DORV, TGA<br>LVOT obstruction<br>Bilat. superior vena cava<br>Interrupted inferior vena cava | | | Anal stenosis | | | 18 |
| Deletion Xq26 | M | Normal situs | TAPVR, LVOT obstruction<br>Bilat superior vena cava | Lumbar and sacral agenesis | | Anal stenosis | Fused kidneys | Aqueductal stenosis with hydrocephalus | 18 |
| Polyalanine expansion | M | Dextroposition of the heart | Bilat superior vena cava | | EA/TF laryngeal atresia | Anal atresia | Multicystic dysplasia | | This report |

M/F Male/Female, ASD Atrial septal defect, AVSD Atrioventricular septal defect, DORV Double outlet right ventricle, EA Esophageal atresia, HLHS Hypoplastic left heart syndrome, LVOT Left ventricular outflow tract, NR, Not reported, TF Tracheo-esophageal fistula, TAPVR Total anomalous pulmonary venous retour, TGA Transposition of the great arteries, VSD Ventricular septal defect

She went into premature labor at 33 weeks, and a male infant with birth weight of 2060 grams (50th centile) was born by ventouse extraction because of failure to progress in the second stage of labor and fetal distress. The male newborn was hypotonic, bradycardic, and showed respiratory distress. Bag valve mask ventilation was not successful as no air entry into the fetal lungs could be established. Also oropharyngeal and nasopharyngeal intubation was not successful because of a blind-ending larynx, distal to the vocal cords. An attempt to put a tube in the proximal esophagus to ventilate the lungs through a possible fistula failed. A tracheotomy was established but nevertheless prolonged efforts to resuscitate were unsuccessful, and the patient died one hour after birth.

External examination showed a proportionate male neonate with anal atresia and a sacral dimple. Postmortem MRI revealed a heart positioned in the right thorax with the apex of the heart towards the left indicating dextroposition of the heart, a persistent left superior vena cava, and myocardial hypertrophy of both ventricles (Figure 1). Both lungs, particularly the left lung, were hypoplastic. A normal trachea with bifurcation was seen, but there existed no connection to the larynx. The distal esophagus was identified, but had no connection to the proximal portion. The right kidney was multicystic and positioned centrally in the lower abdominal region. The left kidney and the spleen showed a normal position and aspect. The rectosigmoid was dilated, as seen in anal atresia. No abnormalities of the vertebral bodies or conus medullaris were observed. MRI of the brain showed normal infra- and supratentorial structures. Autopsy confirmed the laryngeal and esophageal atresia. A low tracheo-esophageal fistula was present. The persistent left superior vena cava was connected to the coronary sinus. No other structural abnormalities of the heart were observed. A centrally located right dysplastic kidney with multiple cysts, and a blind-ending rectum with a possible recto-urethral fistula were found. Autopsy of the brain showed no abnormalities. A fibroblast culture showed normal chromosomal breakage after diepoxybutane (DEB) exposure, making Fanconi anemia unlikely. Dysmorphologic examination of the parents was normal.

## Results

Bi-directional sequencing of all coding exons of the *ZIC3* gene did not reveal mutations, apart from a 6-nucleotide insertion within the portion of exon 1 that codes for a polyalanine repeat. The insertion sequence, GCCGCC, maintains the wild-type reading frame and codes for two additional alanines. The repeat therefore contained 12 instead of the normal 10 polyalanines. The polyalanine sequence of the mother of the patient was normal, and the GCCGCC insertion

was not present, which suggests that the repeat amplification is *de novo* in the affected patient. Randomly selected controls, 44 males and 146 females, from the ethnically-matched population (Dutch Caucasian) were screened for variation in length of this ZIC3 polyalanine repeat. All 336 X-chromosomes showed a wild-type length of 10 repeats of the ZIC3 polyalanine repeat.

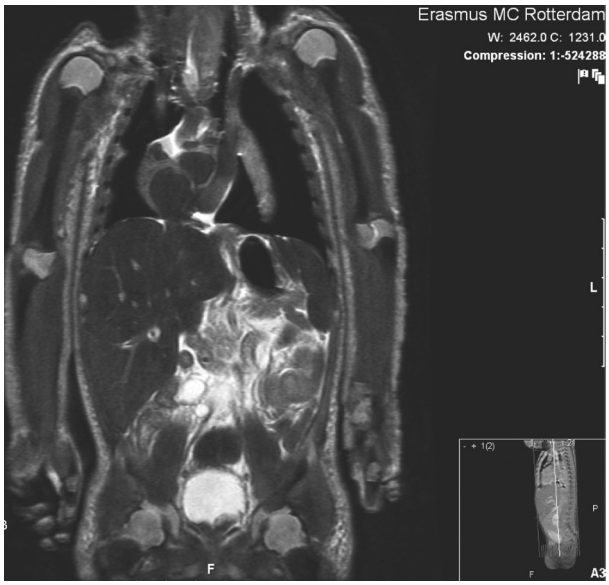



**Figure 1: Postmortem coronal body MRI** showing dextroposition of the heart, persistent left superior vena cava and normal position of liver stomach and spleen. The stomach is filled with air and pneumothorax is present after reanimation

## Discussion

We describe a male neonate with a combination of features resembling the VACTERL association, including anal atresia, esophageal atresia with tracheo-esophageal fistula and a unilateral multicystic kidney. Dextroposition of the heart (heart positioned in the right thorax with the apex of the heart towards the left), with persistent left vena cava, abnormalities not typically seen in VACTERL, were also present. As this phenotype shared characteristics with ZIC3-associated heterotaxy, the *ZIC3* gene (HTX1, MIM 306955) was analyzed and a *de novo ZIC3* mutation was found.

VACTERL association represents a spectrum of anomalies including vertebral defects, anal atresia, esophageal atresia and/or tracheo-esophageal fistula, renal malformations and predominantly preaxial limb anomalies[11-13]. This broad association has considerable overlap with other syndromes including X-linked heterotaxy due to a *ZIC3* mutation. X-linked heterotaxy typically presents with heart defects characteristic of heterotaxy including common atrioventricular (AV) canal, double outlet right ventricle, transposition of the great arteries and abnormal pulmonary or systemic venous connection. Dextrocardia, indicating a mirror image position of the heart, is present in some patients[1,5]. In our patient dextroposition of the heart was found, which could be a result of displacement of the heart, as can be seen in patients with lung hypoplasia. Intestinal malrotation, symmetric liver, abnormal lung lobulation, asplenia or polysplenia are frequently seen in patients with X-linked heterotaxy. Also midline malformations are common, and include imperforate anus, rectal stenosis, sacral agenesis, meningomyelocele, cerebellar hypoplasia, and arhinencephaly[3,5]. X-linked heterotaxy shares anorectal, renal and vertebral malformations with VACTERL association (Table 1). However, laterality defects are rarely found in patients with VACTERL association[11]. Nevertheless, all separate malformations within the VACTERL association have been reported in patients with laterality defects. In two large studies of patients with esophageal atresia and tracheo-esophageal fistula, dextrocardia was reported in 3% of patients[12,14]. Esophageal atresia can also be associated with other features of heterotaxy such as gut malrotation[12,15] and situs inversus[16,17]. Esophageal atresia and tracheo-esophageal fistula are common in VACTERL association, but not in X-linked heterotaxy. Two patients that were initially diagnosed with VACTERL association were later shown to have a *ZIC3* mutation[5,18]. The first patient, described by Purandare *et al.*[18] showed features of VACTERL, including lumbar and sacral agenesis, anal stenosis, fused kidneys and aqueductal stenosis with hydrocephalus without thoracic or abdominal situs anomalies. This patient did have a heart malformation consistent with heterotaxy including total abnormal pulmonary venous return and bilateral superior vena cava[18]. The other patient had VACTERL association with typical vertebral, anal and renal malformation in combination with tracheo-esophageal fistula, but also dextrocardia, congenital heart malformations and asplenia[5]. The anomalies present in these two VACTERL patients are very similar to those of the patient described here. The combination of the VACTERL association with heterotaxy is therefore suggestive of a *ZIC3* mutation, certainly if the disorder is X-linked (Table 1). The presence of tracheo-laryngeal and/or esophageal anomalies in these 3 patients suggests that *ZIC3* plays a role in the development of the foregut.

The majority of *ZIC3* mutations reported to date consist of point mutations (nonsense, missense) or small insertion-deletions resulting in a frameshift, although rarely whole-gene

deletions and X-autosome translocations have been described (Table 1)[2,3,5,19]. In our patient an insertion of GCCGCC in exon 1 of the *ZIC3* gene was identified. This mutation maintains the wild-type reading frame and codes for two additional alanines in the polyalanine stretch of ZIC3. The polyalanine stretch of ZIC3 is an almost perfect repeat where all alanines are encoded by GCC with the exception of the ninth alanine, which is encoded by GCT. As in some dynamic diseases repeat amplification occurs after transition of an incomplete to a complete repeat, we sequenced the alanine repeat from the mother of the patient, but the ninth repeat was normal (GCT). The *ZIC3* repeat amplification is a novel mutation, not reported before neither in patients nor in controls. It is a *de novo* mutation as it was not present in the mother, whereas the patient is also the only affected family member. All randomly selected controls (336 X-chromosomes) from an ethnically-matched Dutch-Caucasian population had 10 *ZIC3* repeats. All together, the *ZIC3* repeat amplification is most likely a disease-causing mutation.

Polyalanine stretches have been predicted in about 500 human proteins, mainly transcription factors. Polyalanine domains in vertebrates are conserved between mammals and the polymorphic nature of sequences coding for polyalanine domains makes them prime candidates for mutations in genetic disorders[20]. Up to now, 9 human diseases have been described that are due to polyalanine stretch elongation[21-23], and X-linked heterotaxy might be the tenth polyalanine repeat disease (Table 2). Nine of these disease genes, including *ZIC3,* encode transcription factors; the only exception is *PABPN1,* a nuclear protein involved in mRNA polyadenylation. Polyalanine stretch elongation of another *ZIC* gene family member *ZIC2* is associated with holoprosencephaly[23-26]. The *ZIC3* repeat expansion described here is the smallest expansion (+ 2 residues) reported, together with the 2-residue polyalanine expansions in *ARX* and *PABPN1* (Table 2). This polyalanine stretch of 12 residues is also the smallest polyalanine stretch reported to be pathogenic, together with the 12-residue polyalanine expansion in *PABPN1.*

Polyalanine tracts expansions in several transcriptions factors, including *HOXD13, HOXA13, RUNX2, SOX3, PHOX2B* and *FOXL2* have been shown to induce cytoplasmatic mislocalisation and aggregation as well as nuclear aggregation[22,27-30]. Several pathogenetic mechanisms have been proposed: (1) Cytoplasmatic aggregation might be dependent upon repeat length as larger oligomers might be unable to pass through the nuclear pores, leading to loss-of-function of the transcription factor as it may not reach the target genes[30]; (2) Intranuclear aggregates might have a toxic gain-of-function affect, as has been suggested for *PABPN1* and *ARX*[31,32]; (3) Aggregation might also induce a dominant-negative effect through the sequestration of the wild type protein or associated proteins in the aggregates. As ZIC and GLI proteins interact, whereby GLI proteins are translocated to the cell nuclei by coexpressed

ZIC3 proteins[33], it is possible that formation of ZIC3 aggregates might also influence the function of ZIC3-related proteins such as GLI3.

In conclusion, we suggest here that *ZIC3* might be the tenth gene implicated in polyalanine expansion diseases, and that *ZIC3* mutations may be present in patients with VACTERL association in combination with heterotaxy.

**Table 2: Polyalanine expansions in human disease**

| Gene | Disease | Expansion |
|---|---|---|
| *ZIC3* | Heterotaxy (HTX1) with VACTERL-like features | 10→12 |
| *ZIC2* | Holoprosencephaly (HPE5) | 15→25 |
| *FOXL2* | Blepharophimosis-ptosis-epicanthus inversus (BPES) | 14→22, 24 |
| *PHOX2B* | Congenital central hypoventilation, Haddad syndrome | 20→25–33 |
| *ARX* | West syndrome, Partington syndrome | 16→18, 23 |
| | | 12→20 |
| *SOX3* | Mental retardation with growth hormone deficiency | 15→26 |
| *RUNX2* | Cleidocranial dysplasia | 17→27 |
| *HOXA13* | Hand–foot–genital syndrome | 14→24, 26 |
| | | 12→18 |
| | | 18→24–30 |
| *HOXD13* | Synpolydactyly | 15→22–25, 29 |
| *PABPN1* | Oculopharyngeal muscular dystrophy | 10→12-17 (AD)<br>10→11 (AR) |

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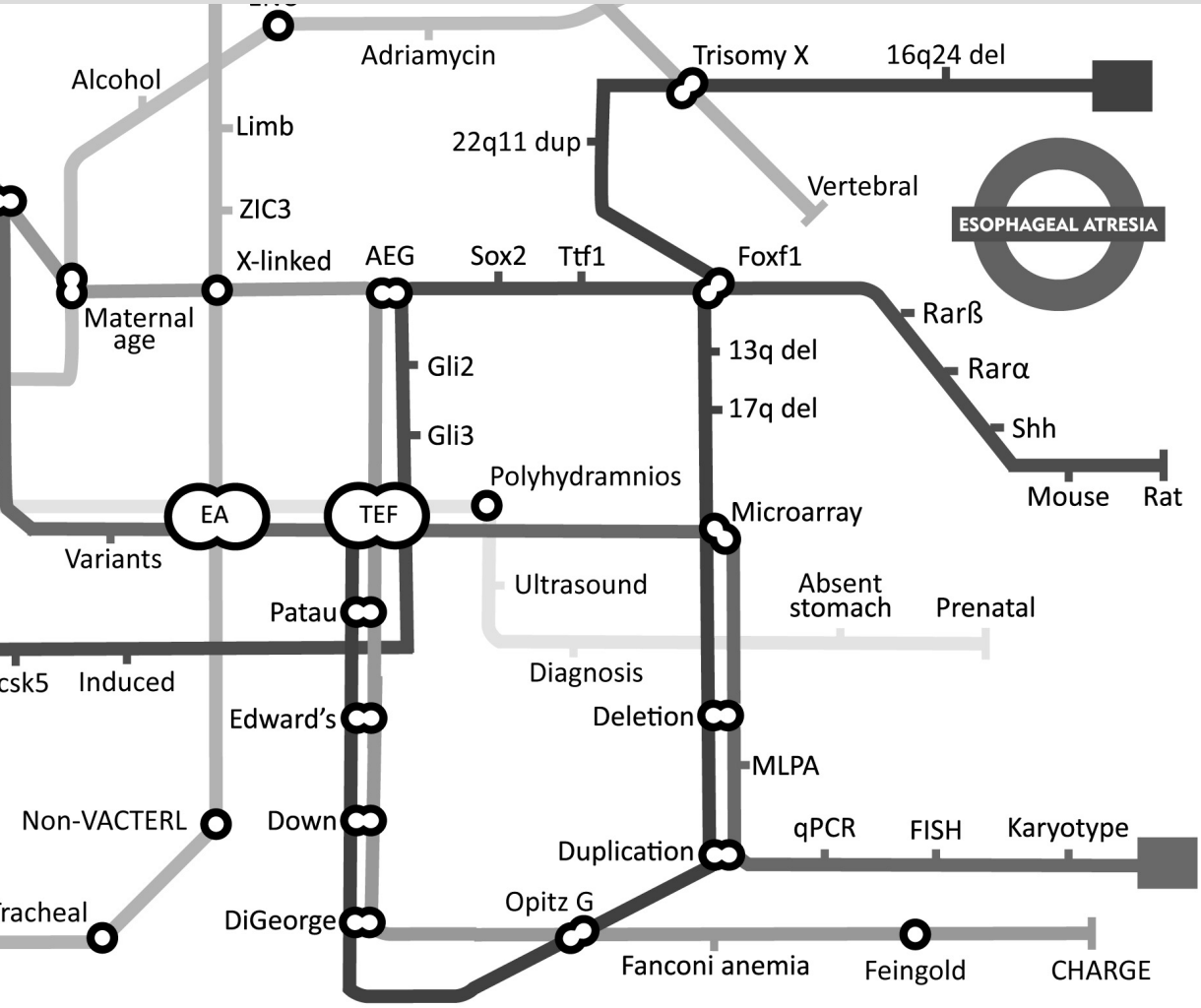
# PART IV

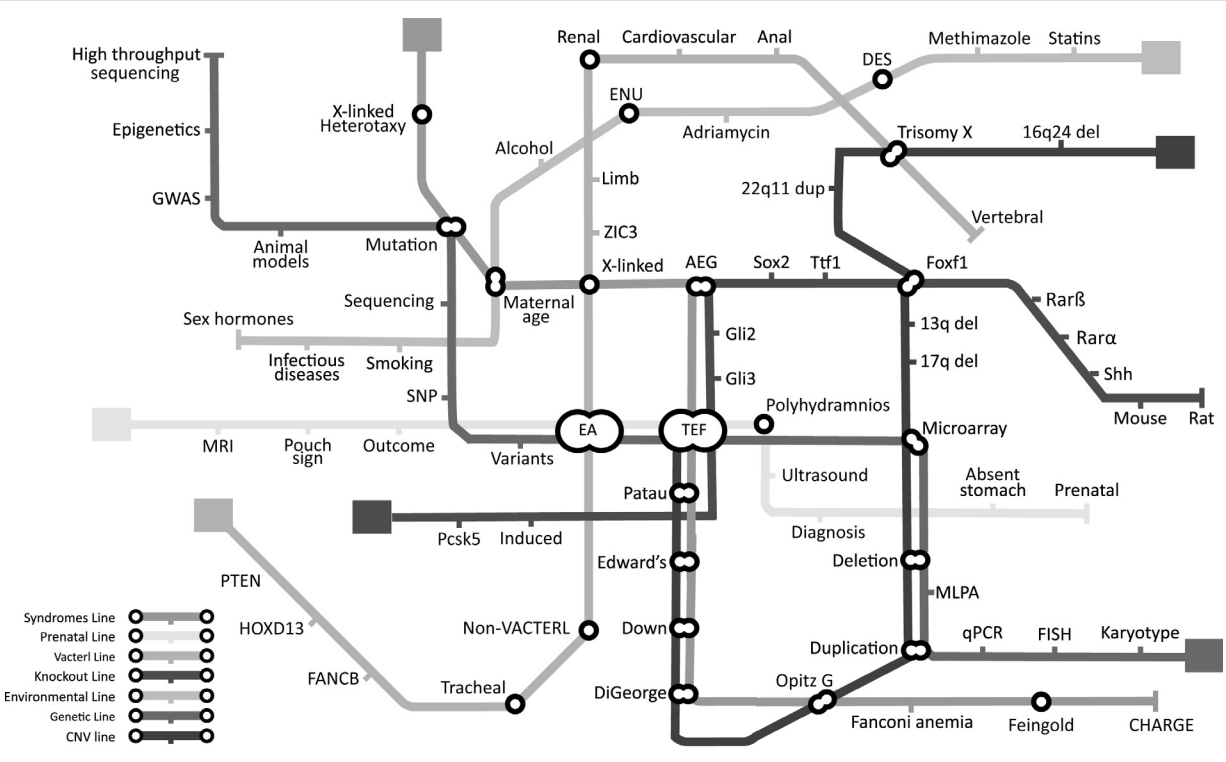
## ‘Mind the Gap’

## Summary and General Discussion



# CHAPTER 9

## Summary and General Discussion




**Adapted from:**

**de Jong EM**, Felix JF, de Klein A ,Tibboel D. 2010. Etiology of Esophageal Atresia and Tracheo-Esophageal Fistula: 'mind the gap'. *Curr Gastroenterol Rep.* 2010 Apr 28. [Epub ahead of print]

# Summary

## Background

**E**sophageal **a**tresia (EA) and **t**racheo-**e**sophageal **f**istula (TEF) are congenital malformations that occur in approximately 1:3,500 live-born infants[1]. Five subtypes are described, based on the location of the atresia and the type of connection between trachea and esophagus[2]. Half of the patients show other anomalies as well, including vertebral, anal, cardiovascular, renal, and limb abnormalities (occurring together in the VACTERL association). As is the case with other congenital malformations, EA/TEF is more prevalent in twins, but usually only one twin is affected[1]. Once a couple has one child with EA/TEF, the risk of having a second child with this anomaly is as high as 1%[3].

Clinical symptoms and ultrasound signs during pregnancy may point at the presence of EA/TEF. However, EA/TEF is most often diagnosed postnatally, because ultrasound signs in relation to EA/TEF are difficult to interpret. We found a significantly higher mortality in prenatally diagnosed infants and also in infants with additional congenital anomalies (Chapter 2). The latter patients are more likely to have experienced a complicated pregnancy[4].

Better surgical techniques and pre- and post-operative care have improved the prognosis of EA/TEF over the last few decades, but patients still have considerable short- and long-term morbidity[5]. These include post-operative complications, esophageal stricture, dysphagia associated with esophageal dysmotility, gastro-esophageal reflux, growth impairment, persistent pulmonary dysfunction, and/or symptoms associated with other major congenital anomalies[5-8].

The pathological mechanism leading to EA/TEF is unknown. The organs involved – trachea, esophagus and lungs – are foregut-derived structures. During the fourth week of embryonic life, the foregut divides into a ventral respiratory part and a dorsal esophageal part. EA/TEF is thought to be a multifactorial complex disease, with involvement of genetic and environmental factors. However, in no more than 6-10% of patients a defined genetic syndrome can be diagnosed[9].

In this thesis we describe the phenotypic (Chapters 2 and 3) and molecular-genetic (Chapters 4-8) approach to EA/TEF and other foregut-related anomalies in the search for more clues to 'bridge the gap' in knowledge on the etiology of EA/TEF.

## Environmental factors

Various environmental factors have been suggested to be risk factors for the development of tracheo-esophageal anomalies, including maternal exposure to methimazole[10], exogenous sex hormones[11], maternal alcohol and smoking[12], infectious diseases[13], and working in agriculture or horticulture[14]. Previous studies implicate a possible role for maternal *in utero* exposure to diethylstilbestrol (DES)[15]. Observations in insulin-dependent diabetic mothers suggest that first trimester exposure to maternal diabetes is associated with the development of congenital anomalies, including EA/TEF and VACTERL-associated anomalies[16]. Very recently, studies from the EUROCAT birth registry network found that older mothers are at significantly greater risk of having a child with EA[17].

Congenital anomalies are more common in twins than singletons but in the majority the etiology remains unknown. EA/TEF is more frequent in twins than in singletons, but the twin concordance rate is low[18,19]. It has been suggested that early loss of a twin conceptus could be a significant risk factor for congenital anomalies[20]. This implicates a possible 'teratogenic environmental' role of twin pregnancies in the development of congenital anomalies. However, how many patients this potentially affects is unknown.

Administration of the anthracycline antibiotic Adriamycin to pregnant rats causes EA/TEF and other major congenital anomalies in the offspring[21]. However, no such association has been reported for humans[22]. A role for vitamin A deficiency in the development of EA/TEF has also been suggested. A vitamin A deficient diet given to pregnant rats caused severe congenital anomalies in the offspring, including agenesis of the lung and TEF[23]. Ethylnitrosourea (ENU), an alkylating and mutagenic agent, induced a recessive mouse mutation with a phenotype that includes abnormal tracheo-esophageal septation and VACTERL-associated anomalies[24]. So far, no specific environmental risk factor has consistently been identified.

## Genetic factors

*Human syndromes and associations involving EA/TEF*

More than half of EA/TEF patients have associated anomalies, notably cardiovascular defects, renal agenesis, microcephaly, duodenal atresia, limb reduction defects, and polycystic kidney[25,26].

More than 30% of EA/TEF cases in our cohort (syndromal cases excluded) were defined as VACTERL-associated (Chapter 3). This means that they showed at least three defects

occurring in the VACTERL association. The use of this acronym as a clinical entity is still being debated, as is the minimum number of defects that must be present. It is known to be a very heterogeneous association.

VACTERL-associated patients are of interest in the search for new genetic factors underlying foregut-related and associated anomalies. However, the combinations of EA/TEF and its associated anomalies resemble and/or overlap phenotypically with defined syndromes, such as CHARGE syndrome, Feingold syndrome, and 22q11 deletion syndrome (discussed below). It is not easy, therefore, to discriminate between the causal syndromes, the more so as the etiology of the VACTERL association is still unclear. Specific phenotypes, such as VACTERL-associated anomalies combined with hydrocephalus, were found to be due to causative mutations in the genes *FANCB* and *PTEN*[27,28]. In addition, causative mutations in the genes *HOXD13, PCSK5* and *ZIC3* (Chapter 8) were found in VACTERL-associated patients[24,29,30]. A recent study found deletions in the *FOX* gene cluster on chromosome 16q24, and mutations in the *FOXF1* gene, in patients with alveolar capillary dysplasia combined with VACTERL-associated anomalies, including EA/TEF[31].

EA/TEF occasionally occurs in combinations with hemifacial microsomia, cardiac, vertebral and/or central nervous system anomalies (Goldenhar syndrome), but no genetic basis has been described for this syndrome[32]. Additionally, an association involving multiple gastro-intestinal atresias (esophageal, duodenal, recto-anal, and biliary atresia), hypoplastic pancreas and gallbladder, intestinal malrotation, and neonatal diabetes mellitus was reported[33-37]. Recently, mutations in the *RFX6* gene were found in patients with neonatal diabetes and small bowel obstruction. With the discovery of this human RFX6 mutation, a phenotypic link with *Rfx6* knockout mice has been made; Rfx6 is required for endoderm and islet development and for the production of insulin in humans and mice[38].

### *Single gene disorders involving EA/TEF*

#### *Feingold syndrome*

Feingold syndrome is caused by germline mutations in, or deletions of, the *MYCN* gene on chromosome 2p24.1. It is the most frequent cause of familial syndromic gastro-intestinal atresias. From 30 to 40% of patients diagnosed with Feingold syndrome have EA/TEF[39]. Marcelis *et al.*[40] reviewed the clinical features of Feingold syndrome in relation to the genotype; 23 different mutations of the *MYCN* gene and 5 deletions encompassing *MYCN* have been described over the years. The authors suggest that the presence of digital anomalies in combination with microcephaly is enough to justify *MYCN* analysis[40]. Such analysis is also recommended in patients with EA/TEF in combination with microcephaly.

*CHARGE syndrome*

The well defined CHARGE syndrome – involving **C**oloboma, **H**eart anomalies, choanal **A**tresia, growth and/or mental **R**etardation, **G**enital and **E**ar anomalies – is in some 60% of cases caused by mutations in the chromodomain helicase DNA-binding (*CHD7*) gene[41]. Around 10% of patients diagnosed with CHARGE syndrome display EA/TEF. The *CDH7* gene is thought to have a role in early embryonic development affecting epigenetic regulation by chromatin organization and euchromatic gene expression. Still, its regulatory function and involvement in CHARGE syndrome and foregut development should be clarified in functional studies in humans and animal models.

*AEG syndrome*

Deletions and mutations of the *SOX2* gene are causative for the phenotype of clinical **A**nophthalmia/optic nerve hypoplasia, **E**sophageal atresia, and/or **G**enital anomalies in the AEG syndrome[42]. Studies in chickens and Xenopus implicate a role for *Sox2* in the developing foregut. Que *et al.* demonstrated EA/TEF in *Sox2* mutant mice and thus provided evidence that down regulation of *Sox2* plays a role in the etiology of EA[43,44]. The proven relation between murine models and the human phenotype was a breakthrough in the knowledge about syndromic EA/TEF.

*Opitz G syndrome*

Opitz G syndrome is characterized by midline abnormalities with mental retardation and agenesis of the corpus callosum. Even though EA/TEF is rare in Opitz G syndrome, the combination of EA/TEF with corpus callosum agenesis warrants testing for mutations in the *MID1* gene, which causes the X-linked form of Opitz syndrome. Laryngotracheo-esophageal defects in general are present in most *MID1* mutated males, but EA/TEF is rare[45]. In addition, deletions on chromosome 22q11.2 cause the autosomal dominant form of Opitz syndrome, which has the same size of the deletion as observed in DiGeorge syndrome[46]. Also several patients with DiGeorge syndrome were reported to have EA/TEF (see below).

*Fanconi anemia*

Fanconi anemia (FA) is a genetically and phenotypically heterogeneous syndrome characterized by progressive bone marrow failure and early occurrence of acute myeloid leukemia, combined with several congenital malformations, including gastro-intestinal malformations. Thirteen genetic subtypes have been described (A, B, C, D1, D2, E, F, G, I, J, L, M, and N), of which *FANCA*, *FANCC,* and *FANCG* are the three commonest disease-causing genes[47]. Mutations in the *FANCA,*

*FANCB*, *FANCC*, *FANCD1 (BRCA2), FANCD2*, and *FANCG* genes have been described in patients with EA/TEF with various combinations of defects, including microcephaly, short stature, pigment changes and several VACTERL-associated anomalies, including heart, renal, and limb defects[27,47-51]. EA/TEF is not a common feature of FA, but gastro-intestinal malformations, including duodenal atresia, anorectal malformations, and EA/TEF, are seen in 14% of all FA patients[47]. In addition, mutations in the *FANCB* gene, on chromosome Xp22.31, were causes of X-linked VACTERL with hydrocephalus in patients who also had EA/TEF, lung lobulation defects, and confirmed central nervous system anomalies[27]. The diagnosis of Fanconi anemia calls for early therapeutic interventions and chromosome breakage studies. Faivre *et al.* advised breakage studies in patients with common VACTERL-associated anomalies, including EA/TEF, in combination with skin pigmentation abnormalities, growth retardation, microcephaly, and/or dysmorphism[48].

*X-linked Heterotaxy (Chapter 8)*

In this thesis, we describe a new single gene disorder related to EA/TEF. In a patient with features of the VACTERL association, including EA/TEF and laterality defects, a 6-nucleotide insertion in the X-linked heterotaxy gene *ZIC3* was found (Chapter 8)[30]. This expands the amino-terminal polyalanine repeat from ten to twelve polyalanines. The clinical picture of the VACTERL association overlaps with X-linked heterotaxy caused by *ZIC3* mutations, as anal atresia and cardiac defects are common in both disorders. Some patients with ZIC3-associated heterotaxy show TEF, renal and vertebral anomalies[52,53].

## *Chromosomal abnormalities*

Many well known chromosomal aberrations are observed in EA/TEF patients, such as Down syndrome (trisomy 21), Edwards syndrome (trisomy 18) and Patau syndrome (trisomy 13)[1]. Several structural chromosomal anomalies were reported and reviewed[54,55]. Deletions on several chromosomal loci, including 22q11 (DiGeorge syndrome), distal 13q, 17q21.3-q24.2, and 16q24.1 are found in some cases[31,54,56-58]. Some of the above-mentioned deletions are of interest, because the deleted loci include several candidate genes for EA/TEF, e.g., the 17q21.3-q24.2 region including *NOG* and *TBX4.* Recently Stankiewicz *et al.* described deletions on the16q24.1 locus, including the forkhead genes *FOXF1*, *FOXC2* and *FOXL1*. Of these transcription factors, *FOXF1* is of particular interest, because heterozygote knockout mice for this protein have EA/TEF[31].

Improved molecular cytogenetic techniques allow mass screening of large cohorts of patients with congenital anomalies; the copy number variations in microarray studies

yield additional deletions and duplications. Microarray studies used in other congenital malformations proved effective in mapping and identifying genes that predispose to the phenotype in an unbiased fashion[59-63]. In studies included in this thesis we used the above techniques to identify possible causative genetic anomalies in EA/TEF patients. We confirmed several chromosomal aberrations in patients with EA/TEF: 22q11 microduplication syndrome (Chapter 4), Triple-X syndrome (Chapter 5), and a 5q11.2 deletion (Chapter 6). In addition, microarray screening in large cohorts of patients has provided more insight into the polymorphic regions of the human genome in relation to inherited aberrations in patients with congenital anomalies (Chapter 7).

### *Murine models*

Several murine models exhibit tracheo-esophageal anomalies (Table 1)[24,44,64]. Genes of developmental pathways are involved, including Vitamin A effectors (*Rarα, Rarβ*), effectors of the sonic hedgehog (*Shh)* pathway (*Shh*, *Gli2*, *Gli3*, *Foxf1*), other homeobox-containing transcription factors and their regulators (*Hoxc4*, *Ttf-1, Pcsk5*), and developmental transcriptional regulators (*Tbx4*, *Sox2*).

Research into the human homologues of these genes has shed some light on the molecular basis underlying some human cases of EA/TEF. For example, Que *et al.* found that 70% of null mutant mice for *Noggin* display EA/TEF. In several human TEF cases there is a deletion of the 17q21.3-24.2 locus, which includes the human orthologue *NOG*[44,58]. Mice haploinsufficient for *Foxf1* may show EA/TEF and other foregut-derived anomalies, including lung hypoplasia and lobulation defects[65]. A patient with a heterozygote deletion on chromosome 16q24, including the *FOXF1* gene, displayed lung anomalies, EA/TEF and other VACTERL-associated features[31]. The *Gli2* and *Gli3* genes are essential for formation of the trachea and the esophagus. Deletions of these genes are associated with congenital defects that resemble the VACTERL association, at least in mice[44,66]. Szumska *et al.* describe an ENU-induced recessive mouse mutation in the *Pcsk5* gene, a regulator of *Hox* genes. Such mutations, in a heterozygote form, were also present in two patients with EA/TEF and features of VACTERL association. However, in these cases the mutations were inherited from a phenotypically normal parent. The etiological role of these mutations remained unclear[24].

**Table 1: Overview of genes essential for tracheo-oesophageal development and their human homologues**

| Gene | Mutant phenotype | Human homologue | Human locus |
|---|---|---|---|
| *Shh*$^{-/-}$ | EA; TEF; lungs form rudimentary sacs | *SHH* | 7q36 |
| *Rarα*$^{-/-}$*;β2*$^{-/-}$ *or Rarα1*$^{-/-}$*;β*$^{-/-}$ | TEF; lung hypoplasia or agenesis | *RARα; RARβ* | *RARα*: 17q21.1; *RARβ*: 3p24 |
| *Gli2*$^{-/-}$*; Gli3*$^{+/-}$ | EA; TEF; severe lung phenotype | *GLI2; GLI3* | *GLI2*: 2q14; *GLI3*: 7p13 |
| *Gli2*$^{-/-}$*; Gli3*$^{-/-}$ | No formation of esophagus, trachea, and lungs | | |
| *Foxf1*$^{-/-}$ | Lethal before embryonic day 10; extra-embryonic defects | *FOXF1* | 16q24.1 |
| *Foxf1*$^{+/-}$ | EA; TEF; lung immaturity/hypoplasia; lobulation defects | *FOXF1* | 16q24.1 |
| *Ttf-1*$^{-/-}$ | TEF; rudimentary peripheral lung primordial | *TTF1* | 14q13 |
| *Sox2* $^{-/-}$ | EA; TEF; lung branching defects | *SOX2* | 3q26.3-q27 |
| *Noggin*$^{-/-}$ | EA; TEF; lung branching defects; abnormal notochord morphogenesis | *NOG* | 17q22 |
| *Pcsk5* C470R mutant* | Abnormal trachea-esophageal septation; hypoplastic lungs | *PCSK5* | 9q21.3 |
| *Hoxc4*$^{-/-}$ | Partially or completely blocked esophageal lumen; disruption of esophageal musculature | *HOXC4* | 12q13.3 |
| *Tbx4* misexpression | TEF | *TBX4* | 17q21-q22 |

Adapted from Felix *et al.*[64], supplemented with findings from recent studies by Szumska *et al.*[24] and Que *et al.*[43,44]. EA, esophageal atresia; TEF, tracheo-esophageal fistula; *, Ethylnitrosourea(ENU)-induced mouse mutation

## Conclusions

In conclusion, EA/TEF is often part of either a spectrum of anomalies in specific syndromes with a known cause or an association. Many genetic pathways have been implicated in the development of EA/TEF. Patients with distinct phenotypes may be diagnosed with genetic syndromes such as Feingold syndrome, AEG syndrome and CHARGE syndrome. There is a substantial gap in our knowledge of how environmental factors combined with genetic factors would disrupt foregut development.

# General Discussion

# ‘Mind the Gap’

Over the last few decades, efforts have been made in understanding the molecular mechanisms of normal and pathological foregut development. They have led to substantial progress in uncovering mutated genes in specific syndromes involving **e**sophageal **a**tresia (EA) and **t**racheo-**e**sophageal **f**istula (TEF). However, in 90% of patients with these congenital malformations the etiology remains unknown.

Overall, it can be assumed that EA/TEF is a clinically, phenotypically, and genetically heterogeneous disorder that can be caused by various factors. So we truly have to work hard at ‘Mind(ing) the gap’. The pediatric surgeon will expertly bridge the physical ‘gap’, but still there remain many ‘gaps’ in knowledge on etiological factors to be bridged by researcher and clinicians. Therefore, in this thesis we tried to ‘mind the gap’ by performing clinical studies focusing on patient phenotyping (Chapters 2 and 3), combining these with targeted molecular genetic studies (Chapters 4-8) in the EA/TEF patient cohort at the Sophia Children’s Hospital.

## Clinical and phenotypical heterogeneity

To illustrate the clinical heterogeneity of EA/TEF, we evaluated the course and outcome for infants either prenatally or postnatally diagnosed with EA/TEF (Chapter 2)[4]. Because ultrasound signs during pregnancy are difficult to interpret, the diagnosis is often made postnatally. Only very recently, efforts to improve the prenatal detection rate have focused on ultrasound or MRI demonstration of the esophageal pouch[67]. From the present level of knowledge we know that certain clinical symptoms and ultrasound signs could suggest the presence of EA/TEF. Our study confirmed that interpretation of these signs is not easy. With regard to postnatal course and outcome, we found that mortality is significantly higher in prenatally diagnosed infants and in infants with additional congenital anomalies. Patients with associated anomalies have more complicated pregnancies with a worse outcome. Parental counseling as to the likelihood of EA/TEF should be undertaken cautiously.

The phenotypic heterogeneity is easily ascertained in our patients, but not easily explained. Generally, associated anomalies are found in over half of EA/TEF patients. Most relevant is the VACTERL association[68]. In Chapter 3[26], we have made a detailed description

of the patients who showed at least three defects included in the VACTERL association. The vertebrae/ribs and the cardiovascular system appeared to be most commonly affected in combination with EA/TEF. In addition, 70% of the patients with VACTERL defects displayed other congenital malformations as well. It is not easy, therefore, to discriminate between the causal syndromes, the more so as the etiology of VACTERL association is still unclear. The phenotype of VACTERL overlaps with several genetic syndromes, such as CHARGE syndrome, Feingold syndrome, 22q11 deletion syndrome and Pallister-Hall syndrome. In clinical practice, syndrome-diagnoses are made on the basis of the phenotypic evaluation of the anomalies present, combined with an approach targeted on known mutations in genes or known loci for microduplication/microduplication syndromes. This approach often remains without results due to the rarity of the syndromes, leaving most of the VACTERL patients without a genetic diagnosis. In addition, clinical practice shows that phenotyping and screening for syndromes is dependent on awareness of the attending clinician. Structured relationships with clinical geneticists result in standardized protocols for further genetic evaluation. However, in the near future more detailed molecular-diagnostic techniques will be available. With this heterogeneous group of patients under investigation, it is likely that some of them can be listed with an identifiable genetic syndrome instead of descriptive term as diagnosis (discussed in detail below).

Standardized clinical and surgical care is necessary to 'bridge the gap' in knowledge on the clinical and phenotypical heterogeneity. This includes an echocardiogram and vertebral and limb X rays, complemented with renal ultrasound evaluation if features of the VACTERL association are present. In addition, EA/TEF patients should be evaluated following standardized genetic protocols for counseling and research. This process should be complemented with the evaluation of possible gene-environment interactions that could provide more insight into new etiological factors[14]. Environmental factors in families of patients with EA/TEF could be further explored by means of structured and validated questionnaires, adding to the knowledge gained from case-control studies. Such studies require large cohorts, as have been used in population-based registries for surveillance of congenital anomalies, such as 'Eurocat' (http://www.eurocat-network.eu/), the 'International Clearinghouse for Birth Defects Surveillance and Research' (http://www.icbdsr.org/), and the 'California Birth Defects Monitoring Program' (http://www.cbdmp.org/).

## Genetic heterogeneity

The term 'genetic heterogeneity' is used when a single disorder, trait, or pattern is caused by genetic factors in some cases and non-genetic factors in others. Similar phenotypes may be caused by mutations in different genes or caused by a chromosomal abnormality. Evidence for genetic heterogeneity in EA/TEF and/or VACTERL association comes from animal models describing the phenotypes[64], and from different defined genetic syndromes[9,19,69].

### *VACTERL association*

Martinez-Frias and colleagues proposed that in the VACTERL association, a 'hit' during blastogenesis influences the entire embryo, which then responds as a coordinated unit, the primary developmental field. This would produce congenital defects in multiple organ systems[70,71]. Mutant animal models could give more clues on the genetic origin of this combination of anomalies. Deletions of the mutant *Gli2* and *Gli3* genes cause defective Shh-signalling and are associated with congenital defects that resemble the VACTERL association[66,72]. In humans, however, mutations involving *Shh* have been associated rather with holoprosencephaly and microphthalmia than with VACTERL defects[73,74].

Szumska *et al.* describe an ENU-induced recessive mouse mutation in the *Pcsk5* gene, a regulator of *Hox* genes. Such mutations, in a heterozygote form, were also present in two patients with EA/TEF and features of VACTERL association[24]. Recently, Cao *et al.* proved in a rat-model that the Gli3 promotor contains two putative Hoxd13 binding sites; it was concluded, therefore, that Gli3 expression is regulated by Hoxd13[75]. Reports on mutations in downstream signaling-molecules of Shh, such as *HOXD13* and *FOXF1,* suggest that these developmental pathways are key to foregut development and associated anomalies[29,76].

A recent study describes deletions of the *FOX* gene (*FOXF1, FOXC2, FOXL1*) cluster on chromosome 16q24, and mutations in the *FOXF1* gene, in patients with alveolar capillary dysplasia (ACD) combined with VACTERL-associated anomalies[31]. The 16q24 candidate region includes the *FOXF1* gene. This gene is a major candidate in foregut development, because *Foxf1* haploinsufficient mice display EA/TEF[65]. The implications of this recent finding and the possible role of *FOXF1* mutations and deletions of the FOX gene cluster, were elegantly reviewed[77].

Interestingly *Foxf1* and *Foxl1* are direct targets of the SHH signalling pathway in the murine developing gastro-intestinal tract. Shh-induced *Fox* expression is depending on Gli2 and Gli3. Additionally, several highly conserved Gli binding sites are located upstream of the *Foxf1* and *Foxl1* promoter sequences[78]. One of the Gli binding sites is located 55kb upstream

of the *Foxf*1 promoter and this might explain why also deletions proximal of the *FOXF*1 gene are associated with a VACTERL-like phenotype in some of the ACD patients[76]. The interactions between several players of the SHH signaling pathway (Patched, Smoothened, Gli2/3 and Foxf1) expressed in different cell types of the developing foregut, could give more clues (Figure 1, adapted from Shaw-Smith, 2009[77]). With different candidate genes in foregut development (SHH, GLI, FOXF1) linked to the 16q24 locus, this is an important candidate region for further research.

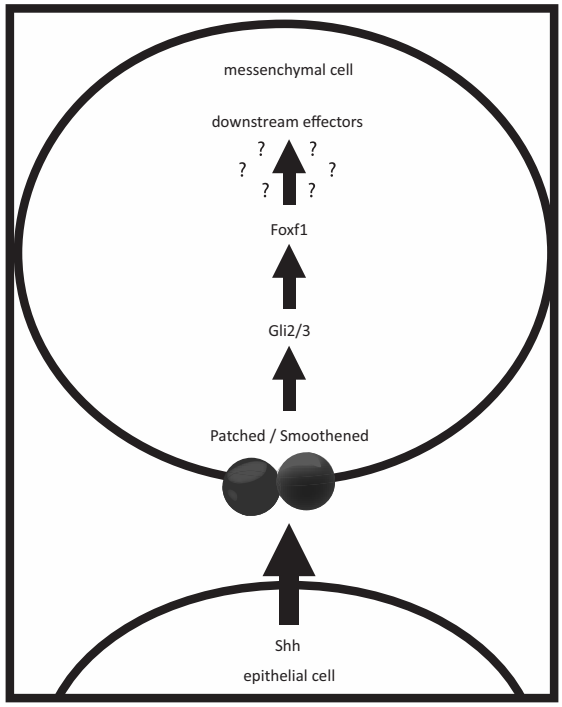



**Figure 1: Epithelial-mesenchymal interactions mediated by the Sonic Hedgehog pathway in the developing foregut.** Adapted from Shaw-Smith[77]

In addition, studies in patients with anorectal malformations implicate a lower expression of SHH, GLI2 and BMP4 in the malformed tissue. Down-regulation of the SHH/BMP4 signaling pathway may be instrumental here, but this could also be the case in foregut-related anomalies[79]. Therefore, it is of interest not only to check blood DNA of the patients with VACTERL-associated anomalies, but also DNA from affected tissue; the proximal pouch or the distal fistula.

The deletions in the *FOX* gene cluster were detected through genome wide screening with microarrays. With microarray data available of over 200 EA/TEF and VACTERL-associated patients, we screened our cohort for possible deletions in the 16q24 region (Chapter 7). This did not reveal any copy number variations of the 16q24 locus. For some of the arrays used, such as the Affymetrix® 250K Nsp, the coverage of the 16q24 region was low and small deletions on this locus could have been missed. In addition, we performed quantitative PCR studies for *FOXF1*, *FOXC2* and *FOXL1* in this cohort to detect possible intragenic deletions. These were found in six EA/TEF patients and two patients with ACD. However, validation with a second technique is indicated. The role of the FOX genes in the SHH–GLI pathway and its downstream targets, in relation to foregut development, ACD and VACTERL-association, should be further explored.

### *Syndromes*

We identified a patient with features of the VACTERL association and a 6-nucleotide insertion in the X-linked heterotaxy gene *ZIC3* (Chapter 8)[30]. This patient's distinct phenotype fits the genetic diagnosis caused by mutations in *ZIC3*. It is of interest that GLI proteins are translocated to the cell nuclei by coexpressed ZIC3 proteins[80]. ZIC3 aggregates might influence the functioning of ZIC3-related proteins such as GLI3, which is a major candidate gene in foregut development, specifically in EA/TEF[66].

As mentioned earlier, the clinical picture of the VACTERL association overlaps with X-linked heterotaxy caused by *ZIC3* mutations. We therefore hypothesized that this novel and *de novo* polyalanine expansion in the *ZIC3* gene contributes to the VACTERL association. To check this hypothesis we screened an additional 25 patients with VACTERL-associated anomalies, without heterotaxy, for ZIC3 mutations. As these were not found, we conclude that mutations in ZIC3 contribute to the heterotaxy and the VACTERL-associated anomalies, but not exclusive for the VACTERL anomalies.

Another interesting feature of the heterogeneity concerns the type of EA (EA+TEF, isolated EA, isolated TEF). None of these types proved correlated to any of the described syndromes. All we can say is that isolated TEF was more frequent in CHARGE syndrome. Perhaps, mutations in the CHD7 gene are associated with different modes of inheritance and influence early developmental pathways related to foregut development[41].



### *Copy number variations*

Microarray studies for other congenital malformations proved effective in mapping and identifying genes that predispose to the phenotype in an unbiased fashion[59-63]. In

collaboration with Baylor College of Medicine, we screened over 200 patients with EA/TEF and foregut-related anomalies for copy number variations (CNVs) (Chapter 7). This approach yielded both *de novo* and inherited CNVs, widely distributed over the genome. Additionally, we found many polymorphic, non-pathogenic variants[81,82]. A few recurrent inherited CNVs were found, specifically on chromosomal loci 5q11, 8p13, 10q25, 12p12 and 22q11. These loci might provide more insight into the polymorphic regions of the human genome in relation to inherited aberrations in patients with congenital anomalies. The origin of non-pathogenic and/or inherited variations and their possible role in the etiology of congenital disease remains unknown.

The degree to which malformations may manifest can be variable in microduplication/microdeletion syndromes. Different causes have been proposed for the variable expression and incomplete penetrance; i.e. other genetic mutations elsewhere in the genome causing the phenotype, compensation of other genes or downregulation of gene targets of the deleted/duplicated genes, epigenetic factors, or a functional SNP on the non-rearranged chromosome could be affected by the genomic rearrangement[83-85]. Recently, a recurrent 520 kb large 16p21.1 microdeletion was described in a cohort of patients with developmental delay. These deletions are often inherited from an unaffected parent, and occur in association with a large CNVs that could explain the variability in these and other microdeletion syndromes[86]. In our cohort, this same inherited deletion was found in a patient with tracheal agenesis and other VACTERL associated anomalies; the 16p12.1 deletion was inherited from his mother, but associated with an additional 5q11.2 deletion of 3.6 Mb[87]. Comparisons with other large cohorts of unaffected controls and affected patients provided evidence of a two-hit model in patients with these inherited 16p12.1 deletions[86]. Clearly this could have profound implications for genetic counseling, especially in the newly discovered microduplication/deletion syndromes[88].

Only a few *de novo* aberrations were found; these included 4q35, 5q11, 6q23, 7p22, 8q13, and chromosome X. Not all regions include (candidate) genes and therefore it is difficult to relate these aberrations to our phenotype. With a genome-wide approach, we found limited *de novo* aberrations and therefore it is unlikely that CNVs, in general, contribute to the phenotype of EA/TEF.

In addition, we checked all human homologues of genes implicated in murine models for possible CNVs. This did not reveal any additional aberrations, but for some genes the coverage of the chosen platforms was limited.

A new approach is in the making however. Data generated from the SNP-platforms will allow performing genome wide association studies, provided patient numbers are such that there is sufficient statistical power.

In a recent pilot study we collected 14 sib-pairs of twins, i.e. 13 discordant and one pair concordant for EA/TEF. Eight pairs were monozygotic and six were dizygotic. Screening for CNVs of both concordant sibs (male/female; dizygotic) revealed an identical 6p22.2 duplication in both. The duplication was confirmed with qPCR. This region includes many histone-genes. Both patients showed the VACTERL association. However, we were not able to determine whether this duplication is inherited from one of the parents, since parental DNA was not yet available. Familial cases of EA/TEF, especially concordant twins, are extremely rare[89-93]. Most concordant twins display the same type of EA/TEF and this seems to indicate that they may have (at least partly) overlapping etiologies.

Findings in twin-studies, e.g. epigenetic differences in both monozygotic and dizygotic twins and our recent finding, can help us to unravel possible genetic and environmental roles in the etiology of congenital malformations[20,94-96]. Therefore, further studies are recommended.

## Strategies and future prospects

*Genotypic approach*

Counseling by a clinical geneticist is advised, including standard karyotyping and screening for subtelomeric aberrations with Multiplex Ligation-dependent Probe Amplification. Clinical data should be reviewed and stored in comprehensive databases, together with genetic information, including pedigrees, DNA, tissue, and cell lines. With improvements in microarray technologies, genome-wide screening for CNVs will replace standard karyotyping in the near future. Diagnostic workflows for patients with congenital malformations and/or mental retardation are being assessed already[97,98].

The importance of detecting possible pathogenic CNVs with a genome wide microarray approach has been proven: haploinsufficiency in dosage-sensitive genes was causative in specific phenotypes and with increasing coverage of the genome the CNVs will become smaller, displaying the true effects on the phenotype. Genome wide microarray studies will change the field of genetic counseling in patients with congenital malformations, because in the future it is expected to be standard applied as a diagnostic tool[99].

Advances in genotyping technology and in knowledge of human genetic variation have enabled genome-wide association studies to identify susceptibility loci for common diseases,

but also for congenital abnormalities, as proven in neural tube defects[100] and syndromic cleft lip/palate[101,102]. This approach, however, requires large cohorts to provide statistical and clinical significance.

Direct sequencing of candidate genes in patients and controls will provide an alternative approach that could reveal low-frequency alleles that influence disease susceptibility (see experimental approach)[103]. In addition, next generation sequencing will soon be an excellent tool in the search for new pathogenic mutations and copy number variations[104-106].

### *Experimental approach*

Studies in animal models implicate an essential role for sonic hedgehog signaling and its downstream effectors during normal development of the foregut. Better understanding of normal and abnormal development of the foregut could be achieved by focusing on the overlapping effectors of *Shh*-signaling, described in patients and animal models, e.g. *Gli2*, *Gli3*, *FOXF1*, *MID1*, *Noggin*, *Bmp4,* and *TBX1*[44,77]. Research into the human homologues of these genes has already shed some light on the molecular basis underlying some human cases of EA/TEF, e.g. for the genes Noggin/NOG[44,58] and Foxf1/FOXF1[31,65]. With the advances in genotyping technology and genome wide direct sequencing, specific candidate genes can be screened for specific mutations. As for EA/TEF and the VACTERL association, several candidate genes of the SHH-pathway are should be screened, including FOXF1, GLI2 and GLI3.

Additionally, there is increasing evidence that epigenetic modifications play an important role in developmental defects[107]. Epigenetic deregulation of germ cells may be caused by certain environmental agents such as diethylstilbestrol[108], pesticides[109], or assisted reproduction technologies[110,111]. Also, mutations in certain genes, already causing a syndromic phenotype, influence epigenetic regulation. An example is the recent finding that mutations the BCOR gene, causing the oculofacialcardiodental syndrome, increase the histone H3K4/36 methylation in mesenchymal stem cells, thereby reactivating transcription of silenced target gene[112]. To the best of our knowledge, there have been no studies specifically studying the role of epigenetic factors in EA/TEF, but it is conceivable that processes such as (de)methylation of genes and imprinting are involved.

## Conclusions

A unifying approach seems to be the answer, therefore analysis of multiple candidate genes should be done in large groups of well-genotyped individuals using next generation high

throughput genomic technology. This will be of great help in detecting possible gene-gene interactions as well as a possible role for CNVs and regulatory mutations in patients with EA/TEF. Then we could sooner or later 'close the gap' in the search for new etiological factors in EA/TEF.

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**Nederlandse samenvatting**
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# Nederlandse samenvatting

## Klinisch en Moleculair-Genetisch Onderzoek bij Oesophagus Atresie

Oesophagusatresie (OA) is een aangeboren afwijking van het spijsverteringskanaal die voorkomt bij ongeveer 1 op de 3,500 pasgeborenen. Er is geen verbinding tussen de slokdarm en de maag. Bij meer dan 85% van de patiënten is een tracheo-oesophageale fistel (TOF) aanwezig. OA/TOF is vaker aanwezig bij tweelingen, waarbij meestal slechts één van de kinderen de afwijking vertoont. Meer dan 50% van de patiënten heeft naast de slokdarmafwijking één of meer structurele aangeboren afwijkingen, bijvoorbeeld afwijkingen aan de wervels, het anorectale kanaal, het hart, de nieren of afwijkingen aan de ledematen. Deze zijn vertegenwoordigd in het spectrum van afwijkingen dat 'VACTERL' genoemd wordt.

In ongeveer 10% van de patiënten met OA/TOF is er een oorzaak bekend voor de afwijking, dit kan gaan om een chromosomale afwijking of een puntmutatie in een bepaald gen. De belangrijkste syndromen beschreven bij OA/TOF patiënten zijn: Down syndroom, Edwards syndroom, AEG syndroom, Feingold syndroom en CHARGE syndroom. Waar voor de rest van de patiënten geen exacte oorzaak bekend is, wordt aangenomen dat er een multifactoriële etiologie is, waarbij zowel omgevingsfactoren als genetische factoren een rol spelen.

In dit proefschrift wordt het onderwerp ingeleid en wordt een overzicht gegeven van bekende oorzakelijke genetische en omgevingsfactoren bij OA/TOF (*Hoofdstuk 1*). Daarna worden de resultaten van klinisch (*Hoofdstukken 2* en *3*) en moleculair-genetisch (*Hoofdstukken 4-8*) onderzoek bij patiënten met OA/TOF gepresenteerd. In het laatste deel (*Hoofdstuk 9*) worden de resultaten samengevat en bediscussieerd.

De relatie tussen prenatale signalen tijdens de zwangerschap (excessieve hoeveelheid vruchtwater en geen of kleine maag zichtbaar op de echo) en postnatale bevindingen bij patiënten met OA/TOF (morbiditeit en mortaliteit) wordt beschreven in *Hoofdstuk 2*. In een retrospectief onderzoek wordt aangetoond dat deze prenatale symptomen negatief gerelateerd zijn aan problemen na de geboorte van een kind met OA/TOF. Wij concluderen dat patiënten met deze prenatale symptomen postnataal meer geassocieerde afwijkingen hebben, en een hogere mortaliteit vertonen. In *Hoofdstuk 3* beschrijven wij de patiënten die, naast OA/TOF, minimaal twee VACTERL-geassocieerde afwijkingen hebben. Bij 23% van alle patiënten in ons cohort bleek dit het geval. Afwijkingen van de wervels/ribben (68.9%) en van het hart (65.6%) komen in deze

groep patiënten het meest voor. Een belangrijke bevinding is dat 70% van deze patiënten ook nog afwijkingen hebben die niet tot het VACTERL spectrum behoren.

In *Hoofdstuk 4* wordt een familiaire vorm van OA/TOF beschreven, gecombineerd met anorectale afwijkingen. De indexpatiënt en zijn niet-aangedane moeder werden gediagnosticeerd met het 22q11 microduplicatie syndroom. OA/TOF is niet eerder beschreven bij dit microduplicatie syndroom. Deze overervende duplicatie (moeder op zoon) bij een familiaire OA/TOF wordt bediscussieerd. In Hoofdstuk 5 worden drie patiënten met het Triple X syndroom en OA/TOF beschreven. Daarnaast werden in ons cohort van patiënten ook twee patiënten met overgeërfde duplicaties van het *SHOX*-gen gevonden. Overexpressie van genen op het X chromosoom zouden wellicht ontwikkelingsprocessen leidend tot aangeboren afwijkingen kunnen beïnvloeden. Een andere X-gebonden afwijking, namelijk een mutatie in het *ZIC3*-gen, wordt later in dit proefschrift beschreven (*Hoofdstuk 8*). Bij een patiënt met heterotaxie, gecombineerd met VACTERL afwijkingen, werd een insertie van 6 nucleotiden in het *ZIC3*-gen geïdentificeerd. Dit leidt tot een polyalanine expansie van 10 naar 12 alanines. Deze nieuw beschreven mutatie in *ZIC3* is naast heterotaxy waarschijnlijk de oorzaak van de VACTERL afwijkingen.

In *Hoofdstuk 6* beschrijven wij een groep van twaalf patiënten met een aangeboren afwijking van de luchtwegen, namelijk luchtpijp-agenesie. Aangezien deze patiënten naast afwijkingen aan de luchtpijp een breed scala aan afwijkingen hebben, zoals VACTERL geassocieerde afwijkingen, zijn deze patiënten moleculair-genetisch gescreend met de array-CGH techniek. Hierbij kwamen meerdere niet-pathogene en overgeërfde afwijkingen aan het licht. Daarnaast werd in één patiënt een 3.6 Mb grote deletie op chromosoom 5 gevonden. De bevindingen van deze studie ondersteunen het heterogene fenotype en genotype dat bij deze patiënten wordt gezien. De cohort studie van alle patiënten met OA/TOF, in samenwerking met het Baylor College of Medicine (Houston, USA), wordt beschreven in *Hoofdstuk 7.* Meer dan 200 patiënten met OA/TOF zijn gescreend op chromosomale veranderingen (deleties en/of duplicaties). Bij alle patiënten zijn afwijkingen gevonden, die niet-pathogeen of overgeërfd waren. De betekenis van de overige afwijkingen is tot op heden moeilijk te bepalen.

In *Hoofdstuk 9* wordt een algemene samenvatting gegeven en worden de resultaten van de studies in dit proefschrift besproken. De huidige stand van kennis wordt in een toekomstperspectief geplaatst, waarbij een interactie tussen klinisch en moleculair-genetisch onderzoek nodig is om de etiologie van OA/TOF verder te ontrafelen.

# Dankwoord

Aan het eind van mijn promotietraject ben ik dank verschuldigd aan velen. Iedereen met wie ik heb samengewerkt en die bij het onderzoek betrokken is geweest, wil ik hartelijk danken.

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Mijn promotor prof.dr. Dick Tibboel. Beste Dick, de treinreis naar Rotterdam voor een stageplek heeft de jaren erna beïnvloed. Dank voor je enthousiasme waarmee je jonge onderzoekers drijft in het doen van wetenschappelijk onderzoek; ik ben er één van. Ik dank je voor alle kansen en mogelijkheden die ik gekregen heb.

Mijn co-promotor dr. Annelies de Klein. Beste Annelies, de mogelijkheid om genetisch onderzoek te doen heb ik vanaf het begin als een uitdaging te gezien. Dankzij jouw kennis en hieraan gelieerd je doeltreffendheid heb ik kunnen groeien in dit project. Dank voor je inzet, kennis en enthousiasme voor de kinderchirurgische projecten! Mijn dank is groot.

De overige leden van de kleine promotiecommissie. Prof.dr. Klaas Bax: dank voor het lezen van het manuscript en specifiek dank voor de betrokkenheid bij de oesophagus atresie patiënten. Prof.dr. Ben Oostra: dank voor mijn plek op de klinische genetica. Ik hoop dat het in de toekomst een gegeven is dat er gezamenlijke promovendi van de Kinderchirurgie en de Klinische genetica zijn. Dr. Charles Shaw-Smith: thank you very much for your time to read and comment my thesis. As an authority in the field of ‘etiology of esophageal atresia’, I am honored to have you in the committee.

Alle leden van de grote promotiecommissie. Prof.dr. Hugo Heij: van wetenschapsstage aan het VUmc Amsterdam naar promotie aan het ErasmusMC Rotterdam. Dank voor alle momenten waarop ik me als ‘import Rotterdammer’ welkom voelde op de OK in Amsterdam. Prof.dr. Ronald de Krijger: vanaf het begin liep ik ‘in’ en ‘uit’ bij de pathologie. Ik ben me er welkom blijven voelen en daarom ben ik blij dat je in mijn grote commissie wilt plaatsnemen. Prof.dr. J. Molenaar: het is een enorme eer dat u wilt plaatsnemen in de grote commissie. Prof.dr. Frans Hazenbroek: ik dank u dat u mijn promotie wilt voorzitten als ‘oud-gediende’ van de afdeling waarop ik met veel plezier heb gewerkt.

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Evelien en Madelein; wat ben ik bevoorrecht met zulke geweldige zussen! Altijd zijn jullie er voor mij en ik zal er altijd voor jullie zijn.

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Werner, 'er loopt een weg…van jouw hart naar het mijne…en terug'. Dank je lieverd, voor alles! Tot de maan en verder…

*Liesbeth*

# Curriculum Vitae

Elisabeth Maaike de Jong was born in Hendrik-Ido-Ambacht, The Netherlands on November 10th 1982. After finishing secondary school at the Kalsbeek College, Woerden in 2000, she started her Medical training at the Free University Amsterdam (VUmc), The Netherlands. She worked as a Professors-assistant and completed additional courses in Medical Ethics at the Metamedica Department, VUmc, Amsterdam. In December 2005, she obtained her Master's degree under the supervision of Professor D. Tibboel, entitled 'VACTERL association in esophageal atresia' at the ErasmusMC – Sophia Children's Hospital. This project was followed by a grant from the Sophia Foundation for Scientific Research, which enabled her to start her PhD degree in May 2006. Under the supervision of Professor D. Tibboel and Associate Professor A. de Klein she has been working on the project entitled 'Genetic studies in Esophageal Atresia and VACTERL association', with the Departments of Pediatric Surgery and Clinical Genetics. The author is continuing her medical training at the ErasmusMC, Rotterdam. She lives together with her partner Werner de Jonge.

# PhD Portfolio Summary

*Summary of PhD training and teaching activities*

| | |
|---|---|
| Name PhD student | Elisabeth Maaike de Jong |
| Erasmus MC Departments | Pediatric Surgery & Clinical Genetics |
| Research School | Medical Genetics Centre South-West Netherlands |
| PhD period | May 16, 2006 – June 9, 2010 |
| Promotor | Prof. dr. D. Tibboel |
| Co-promotor | Dr. J.E.M.M. de Klein |

**1. PhD training**

| | Year | Workload (hours) |
|---|---|---|
| **General academic skills** | | |
| – Biomedical English Writing and Communication | 2008 | 80 |
| – Research Integrity | 2008 | 10 |
| **Research skills** | | |
| – Classical methods for Data-analysis (CCO2) | 2007 | 160 |
| – Reading and Discussing Literature in Molecular and Cell Biology | 2006 | 56 |
| – Discussions & Presentations | 2006 | 25 |
| – Safe Laboratory Techniques | 2006 | 6 |
| **In-depth courses** | | |
| – Master course 'Molecular and Cell Biology (MCB)' | 2007 | 168 |
| – SNP's and Human diseases Course IV (Molmed) | 2007 | 40 |
| – Biomedical Research Techniques V (Molmed) | 2006 | 40 |
| – (Advanced) Browsing Genes and Genomes with Ensembl (Molmed) | 2007-2008 | 16 |
| – Applied Bioinformatics 'Finding your way in biological information' (Molmed) | 2008 | 8 |
| – Molecular Diagnostics II | 2007 | 40 |
| – From Development to Disease (MGC) | 2008 | 40 |
| – Nexus (Molmed) | 2009 | 16 |

| 1. PhD training | Year | Workload (hours) |
|---|---|---|
| **(Inter)national conferences** | | |
| – American Society of Human Genetics Annual Meeting (poster contribution) | 2008 | 40 |
| – Wetenschapssymposium Emma Kinderziekenhuis/AMC Amsterdam (poster contribution) | 2008 | 8 |
| – 'Genomic Disorders' Hinxton, England (poster contribution) | 2008-2009 | 64 |
| **Seminars and workshops** | | |
| – MGC PhD workshops; Maastricht – Heidelberg – Brugge | 2007-2009 | 90 |
| – Genetica Retraite, Kerkrade | 2007-2008 | 30 |
| – Eurocat Jubileum Symposium 'Past, Present & Future' | 2007 | 8 |
| – Next Generation Sequencing (Molmed) | 2007 | 8 |
| – Presentations collaborators; | | |
| Kinderchirurgisch Centrum Amsterdam (KCA), NL | 2007-2009 | 10 |
| John Radcliffe Hospital, University of Oxford, England | 2009 | 10 |
| VOKS support group for EA/TEF parents, NL | 2006-2009 | 10 |

| 2. Teaching activities | | |
|---|---|---|
| **Lecturing** | | |
| Keuzevak 'Pediatric Surgery'; 3rd Year-classes, Medicine, Erasmus MC – Sophia Children's Hospital | 2006-2009 | 10 |
| **Supervising Master's theses** | March-Sept | |
| 'Aetiology of Esophageal Atresia; What about FOX?' Joyce Reijnierse | 2009 | 7 months |

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D'Haene B, Hellemans J, Craen M, De Schepper J, Devriendt K, Fryns JP, Keymolen K, Debals E, de Klein A, **de Jong EM**, Segers K, De Paepe A, Mortier G, Vandesompele J, De Baere E. Improved Molecular Diagnostics of Idiopathic Short Stature and Allied Disorders: Quantitative Polymerase Chain Reaction-Based Copy Number Profiling of SHOX and Pseudoautosomal Region 1. *Clin Endocrinol Metab* 2010 Apr 7 [Epub ahead of print].

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**de Jong EM**, Poddighe PJ, Sloots CEJ, Torfs CP, De Klein A, Brooks AS. Familial esophageal atresia and anorectal malformation; a causative role for a 22q11 microduplication or a chance occurrence? Submitted.

Felix JF, **de Jong EM**, Torfs CP, de Klein A, Rottier RJ, Tibboel D. 2009. Genetic and environmental factors in the etiology of esophageal atresia and/or tracheoesophageal fistula: an overview of the current concepts. *Birth Defects Res A Clin Mol Teratol* 85:747-754.

Felix JF, de Munck AA, **de Jong EM**, Lodder EM, Swagemakers S, de Krijger RR, van der Spek PJ, Tibboel D, de Klein A, Rottier RJ (2007). Gene-expression analysis of tracheo-oesophageal fistulas. Aetiological Studies in Oesophageal Atresia/Tracheo-Oesophageal Fistula: A combined genetic and environmental approach. Rotterdam, PhD Thesis J.F. Felix**:** 87-113.

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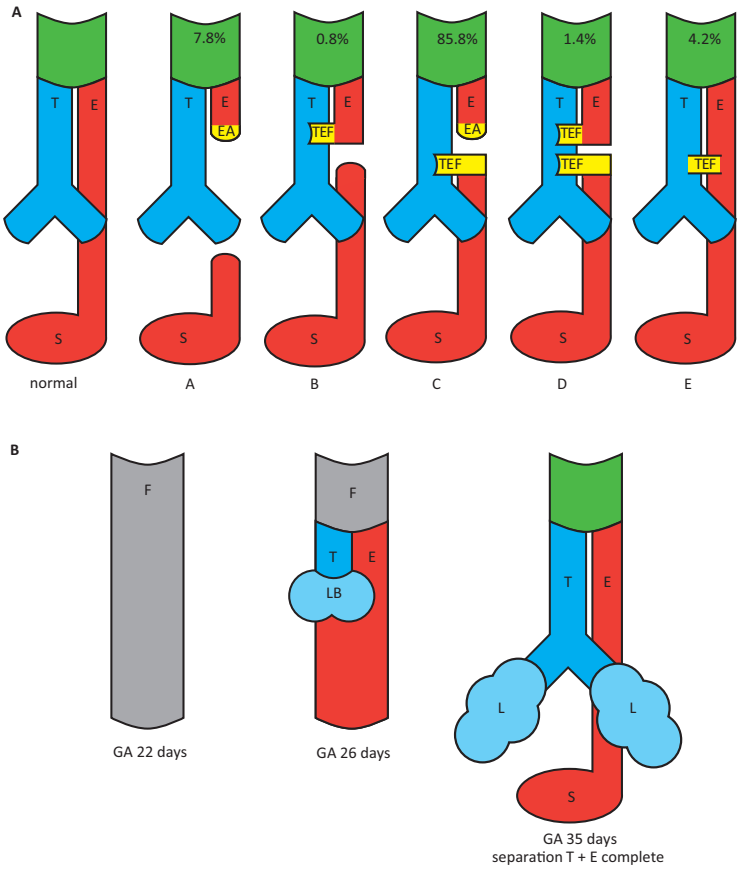
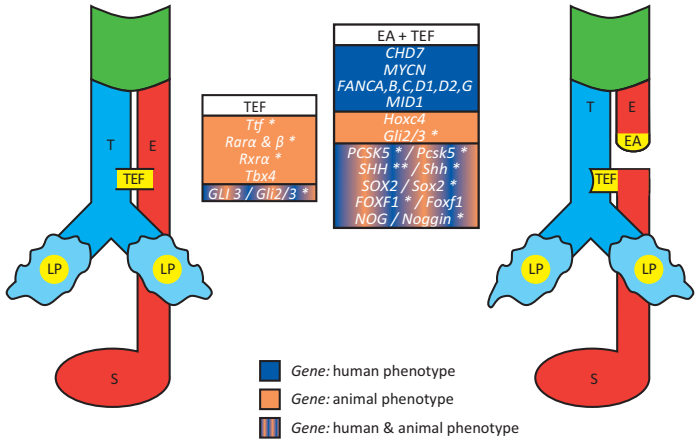
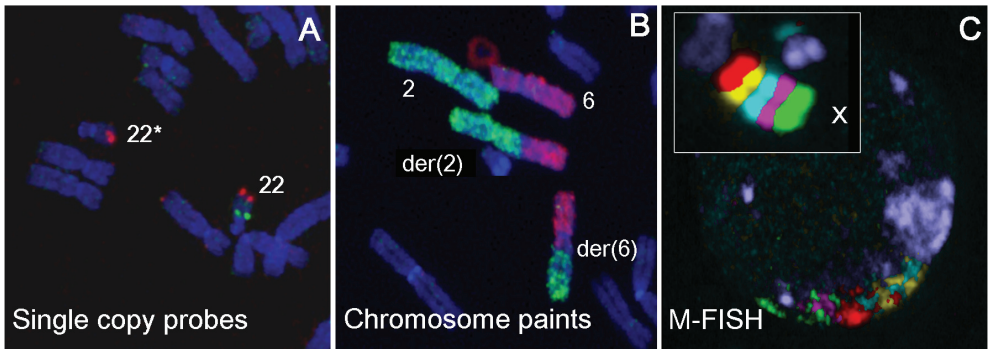
# Color figures




**Chapter 1: Figure 1** *(see page number 13)*




**Chapter 1: Figure 2** *(see page number 22)*

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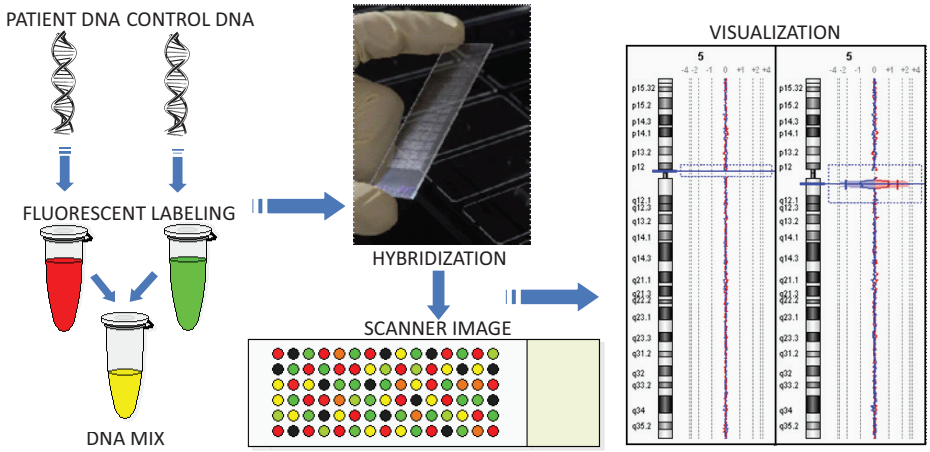


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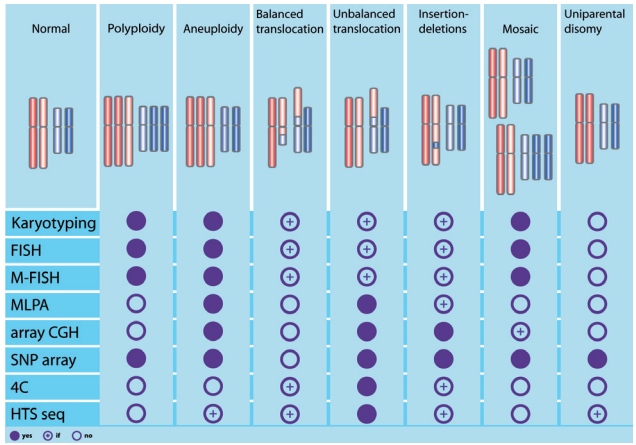


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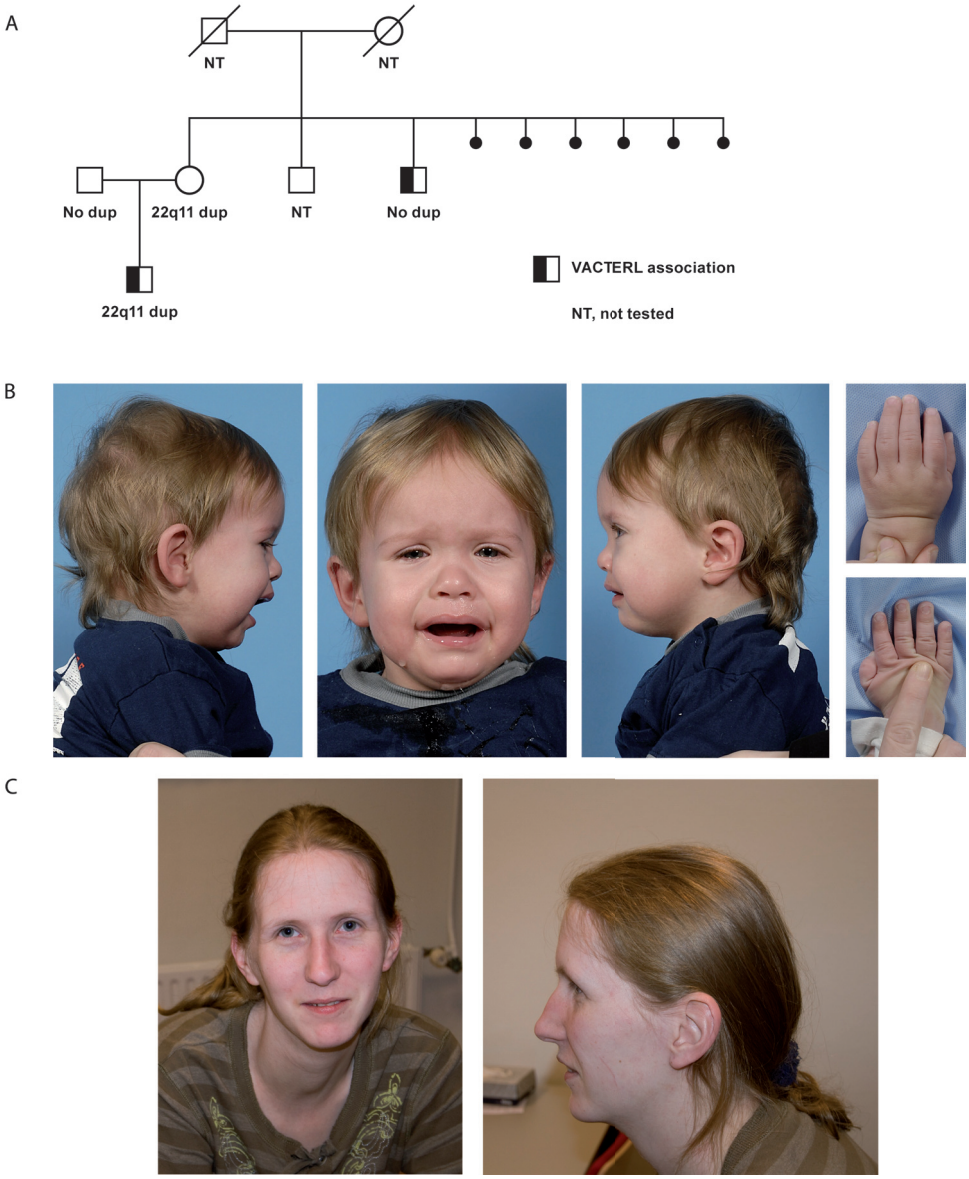
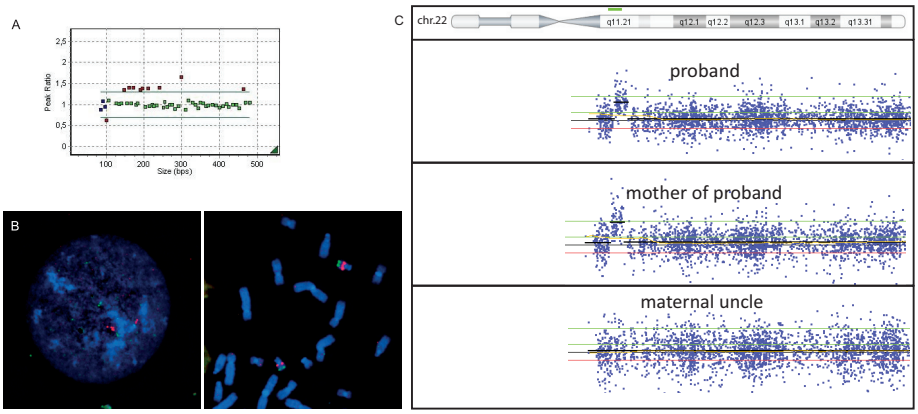



**Chapter 4: Figure 1** *(see page number 76)*

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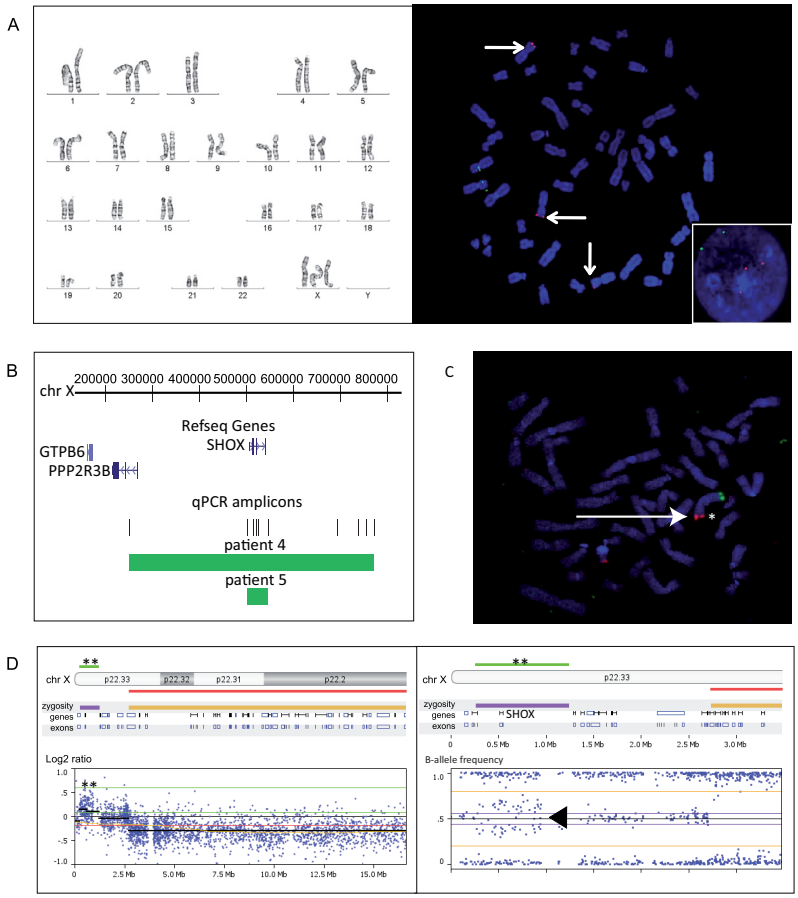


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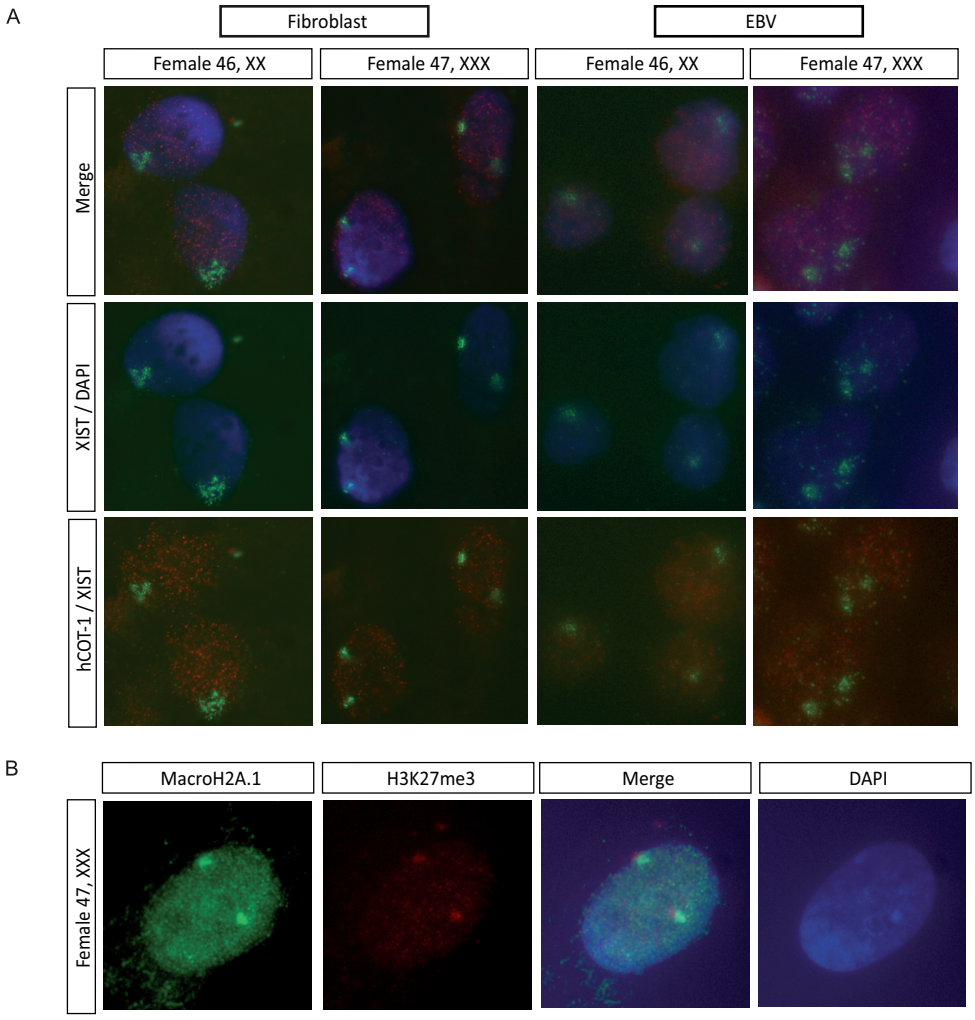


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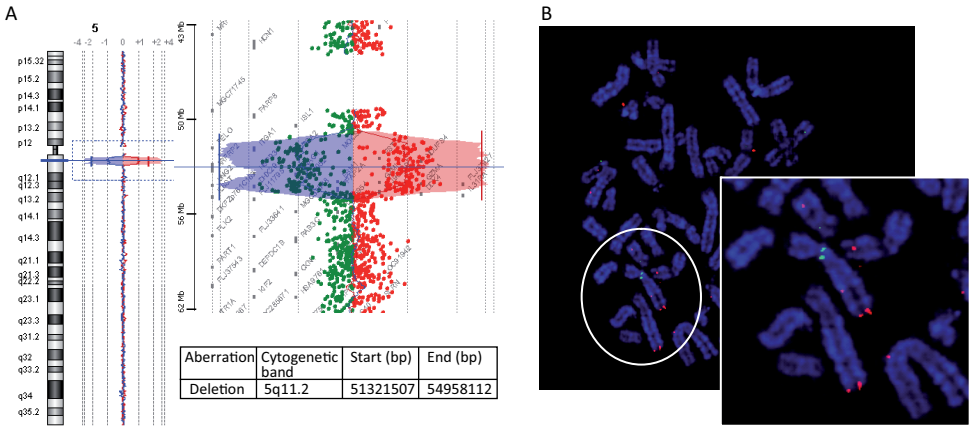
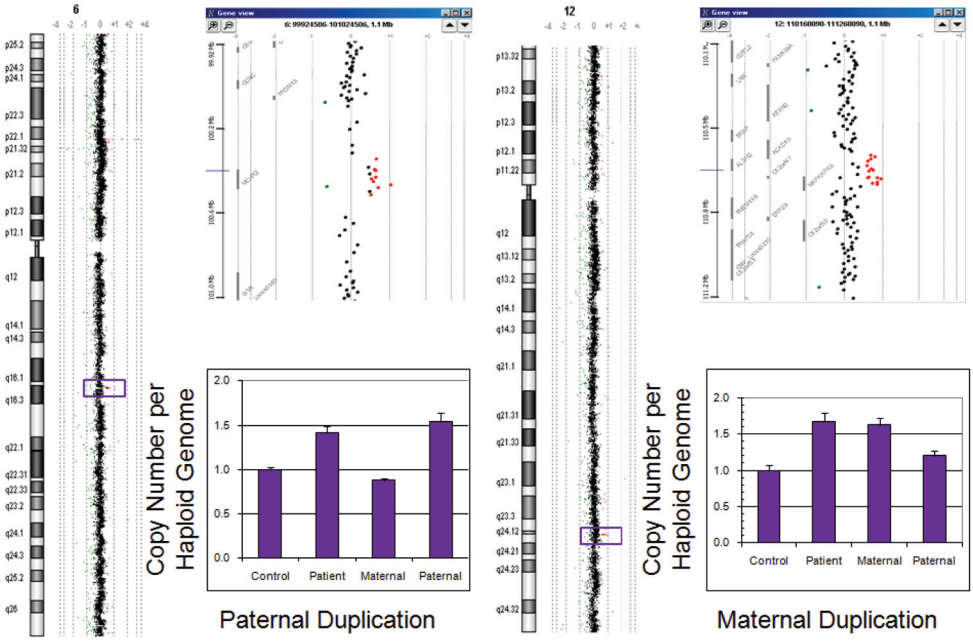



**Chapter 6: Figure 1** *(see page number 108)*




**Chapter 7: Figure 1** *(see page number 122)*