Aetiological Studies in Oesophageal Atresia / Tracheo-Oesophageal Fistula
A combined genetic and environmental approach

Etiologisch onderzoek naar oesophagusatresie / tracheo-oesophageale fistel
Een gecombineerde genetische en omgevingsgerichte benadering

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He who adds not to his learning diminishes it.

The Talmud

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GENERAL INTRODUCTION

Congenital Anomalies

Congenital anomalies are a cause of significant morbidity and mortality in children. Prevalence rates in the literature range from 2.5 to 6.9%, depending on inclusion criteria and surveillance systems.[1-6] The best estimate for the Netherlands comes from EUROCAT Northern Netherlands and shows defects in 2.6% of all births.[3] Half of these of children have more than one congenital anomaly, and in some cases a syndrome diagnosis can be made.[5]

Only a limited number of birth defects have a recognizable cause. Some 13 - 14% are monogenic or are caused by a specific chromosomal anomaly, 6 - 7% spring from environmental causes, including maternal disease, and around 20% are multifactorial. Still, in some 60% of cases, the exact cause of the congenital anomaly is not yet known.[5]
In the Netherlands, with an annual birth rate of around 200,000, approximately 5,200 children are born each year with a birth defect, with severities ranging from very minor to life threatening. Seven severe congenital anomalies that require short-term surgical intervention are sometimes referred to as index diagnoses. These are: congenital diaphragmatic hernia, anorectal malformations, small bowel atresias, gastroschisis, omphalocoele, Hirschsprung disease and oesophageal atresia/tracheo-oesophageal fistula.[7] Children with these diagnoses are treated in one of the six paediatric surgical centres in the country.

Oesophageal atresia (OA) with or without tracheo-oesophageal fistula (TOF) is a developmental defect of the oesophagus that typically results in lack of continuity of the upper gastro-intestinal tract. This causes severe problems soon after birth, including the inability to feed and risk of respiratory complications.

Epidemiology

Oesophageal atresia, either with or without tracheo-oesophageal fistula occurs in around 1 in 3,500 newborns.[4,8-10] Boys are slightly more often affected than girls, with reported male:female ratios ranging from 1.12 to 1.63.[8-10]

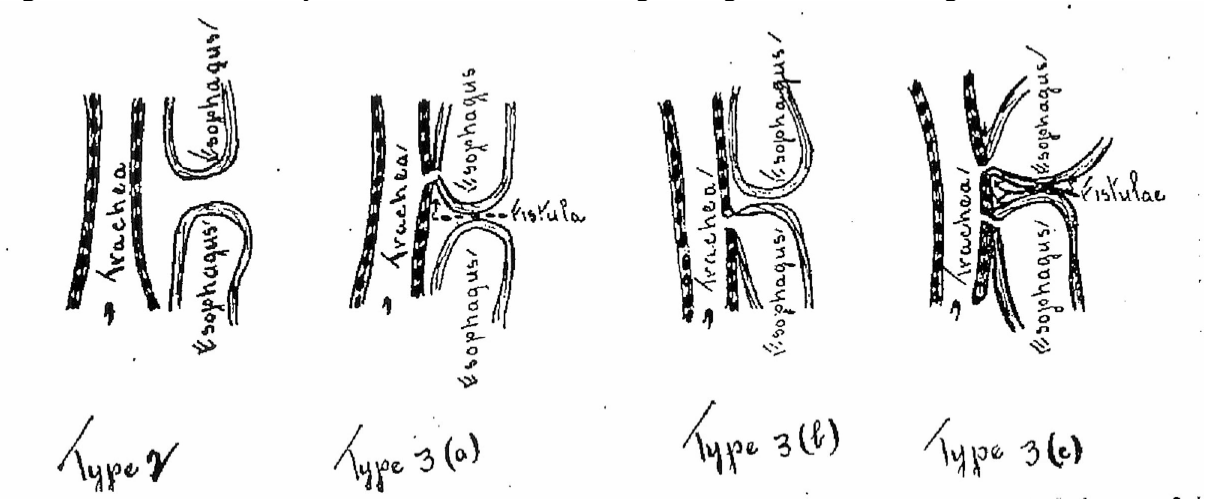
Two main classification systems are in place, one according to Vogt and one according to Gross.[11,12] Details are shown in Table 1 and Figure 1. Gross type B and Vogt type 3a, atresia with a proximal fistula, is depicted most accurately by Vogt, as the fistula is not usually at the tip of the proximal oesophagus, but rather somewhat more cranially. Often, however, a descriptive approach is used.
The most frequent type is proximal atresia with a distal fistula (85.8%), followed by pure oesophageal atresia (7.8%), H-type tracheo-oesophageal fistula (4.2%), atresia with both a proximal and a distal fistula (1.4%) and atresia with a proximal fistula (0.8%).[13]
Associated anomalies are found in around 50% of cases. Of these, cardiac malformations are commonest (up to 24% of all cases), followed by anorectal defects (10 - 14%).[10,14]

Table 1 Classification systems for OA/TOF

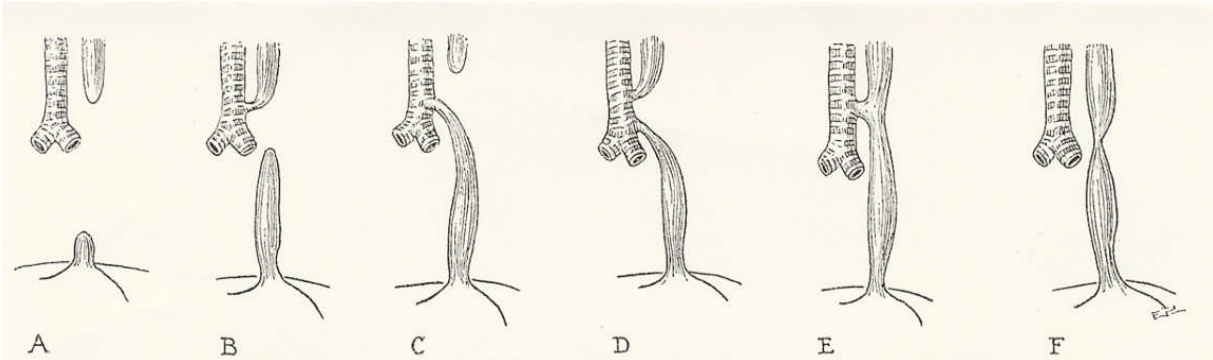
Type	Vogt classification	Gross classification
Total absence of oesophagus	1	A
OA without TOF	2	A
OA with proximal TOF	3a	B
OA with distal TOF	3b	C
OA with proximal and distal TOF	3c	D
H-type TOF	4	E
Oesophageal stenosis	N/a	F

N/a: non-applicable

Figure 1 Classification systems for OA/TOF according to Vogt (a) and according to Gross (b)



a *Type 1 (total absence of the oesophagus) and type 4 (H-type TOF) are not shown. See also table 1. (Reprinted with permission from the American Journal of Roentgenology)*



b *(Reprinted from: Gross RE, Surgery of Infancy and Childhood, page 76, Copyright 1953, with permission from Elsevier)*

Several syndromes and associations have OA/TOF as a feature (Table 2). The best known association of anomalies is the VACTERL (Vertebral, Anal, Cardiac, Tracheo-oEsophageal, Renal, Limb) association and is seen in 10 - 30% of infants with OA/TOF.[9,14] Advances in surgical techniques and perioperative care have stepped up survival rates to over 90% for isolated cases. Lower survival rates are reported for patients with multiple anomalies, often due to severe cardiac anomalies.[8,15,16]

The twinning rate among children with OA/TOF (3.5 - 5%) is significantly higher than that in the general population (1 - 2%).[8-10] It is possible that an error in early embryogenesis which causes the twinning process may still exert an influence a few weeks later and give rise to the defect. Alternatively, the twinning process itself may disrupt early 'organizers' of foregut development or it may cause a global cellular deficiency from which both twins catch up to a certain degree, but not completely, leading to defects in the development of specific organs.[17]

Together with rarer anomalies such as tracheal agenesis and laryngotracheo-oesophageal cleft, oesophageal atresia and tracheo-oesophageal fistula can be seen as a spectrum of anomalies of foregut development. With a reported incidence of one in 50,000 live births, tracheal agenesis is very rare.[18] Nevertheless, studying the development and aetiology of rare, related anomalies may help to gain information about abnormal foregut development and possible causes of defects such as OA/TOF.

History

The first description of a child with oesophageal atresia dates back to 1670, when William Durston reported a set of conjoined twins in his "A narrative of a monstrous birth in Plymouth October 22, 1670; together with the anatomical observations taken thereupon by William Durston, Doctor of Physick, and communicated to Dr. Tim Clerk". At autopsy, Durston noticed that the "oesophagus from the mouth of the right head descended no lower than a little above half an inch off the midriff, and there it ended", thereby describing one of the rarer forms of oesophageal atresia, that without a fistula to the respiratory tract.[19]

Credit for the first report of the most common form of oesophageal atresia, that with a blind-ending proximal pouch and a distal oesophageal part fistulizing to the trachea, should be given to Thomas Gibson, who in 1697 described this finding at autopsy of a child who had died on the third day of life.[20]

The next two centuries brought a number of case reports, invariably with fatal outcome (for overview, see ref. 21). In 1888, the first attempt at surgical correction was made by Charles Steele, who had diagnosed an obstruction of the oesophagus in a newborn. Not sure if it was a membrane or a blind termination, he decided to surgically explore the defect. Upon finding of a true atresia and with no means for repair, he had no choice but to end the operation, after which the child died.[22]

Table 2 Syndromes and associations in which OA/TOF has been described

Syndrome/association	Gene involved	Chromosomal locus	OMIM	References
Frequently associated with OA/TOF (multiple cases described)				
VACTERL association	N/a	N/a	192350	23
VACTERL association with hydrocephalus (VACTERL-H)	PTEN[a] or X-linked	10q23.31 X-linked	276950 314390	24 - 26
AEG syndrome	SOX2	3q26.3-q27	206900	27
CHARGE syndrome	CHD7 or SEMA3E[b]	8q12.1 7q21.1	214800	28 - 32
Feingold syndrome	MYCN	2p24.1	164280	33, 34
X-linked Opitz syndrome	MID1	Xp22	300000	35 - 38
22q11 deletion syndrome	TBX1[b]	22q11.2	188400	39 - 44
Goldenhar syndrome	N/a	14q32	164210	45 - 51
Rarely associated with OA/TOF (a few cases described)				
Pallister-Hall syndrome	GLI3[b]	7p13	146510	39, 52 - 54
Fanconi anaemia	FANCA or FANCC	16q24.3 9q22.3	227650 227645	55, 56
Multiple gastro-intestinal abnormalities	N/a	N/a	601346	57 - 58

Syndromes and associations in which OA/TOF is described in only a single case are not included in the table.
[a] Mutation found in 1 case with VACTERL-H, including OA/TOF
[b] No mutations in this gene described in patients with OA/TOF
VACTERL: Vertebral, Anal, Cardiac, Tracheo-oEsophageal, Renal and Limb anomalies; N/a: not applicable; AEG: Anophthalmia-Esophageal-Genital; CHARGE: Coloboma, Heart anomaly, Choanal atresia, Retardation, Genital and Ear anomalies

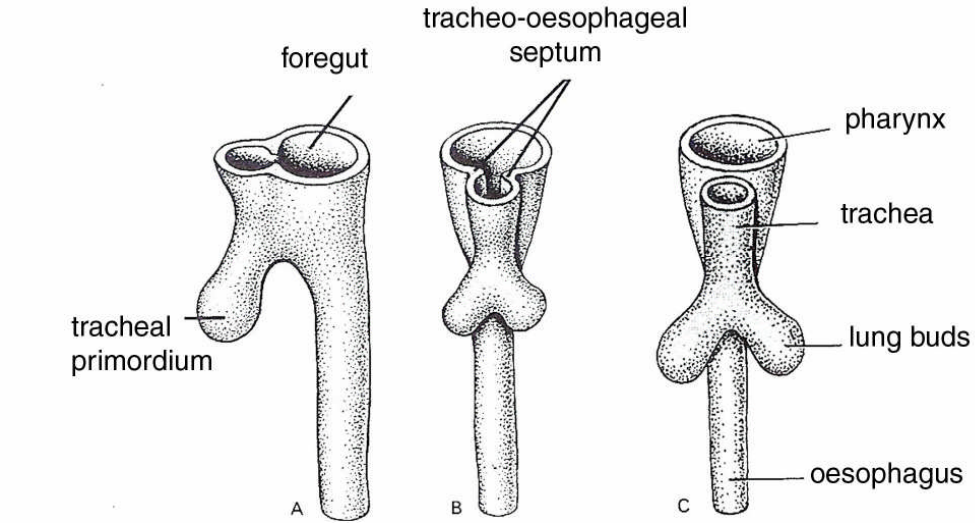
In 1935, a child with oesophageal atresia without a tracheo-oesophageal fistula was the first to survive this anomaly, albeit with a gastrostomy until the age of 16, when jejunal interposition was performed.[59] Leven and Ladd, both in the early 1940s, successfully performed a multi-staged repair of a proximal oesophageal atresia with a distal tracheo-oesophageal fistula.[60,61] The first successful primary anastomosis with long-term survival is credited to Cameron Haight in 1941,[62] which marked the start of an era in which oesophageal atresia changed from a uniformly fatal condition to a severe, yet basically operable congenial malformation.

Embryology

The exact mechanisms of normal and abnormal development of the foregut are still being debated. During the fourth week of embryonic life, the foregut endoderm differentiates into a ventral respiratory part and a dorsal oesophageal part. The laryngotracheal diverticulum evaginates from the ventral side of the foregut and grows into the surrounding mesenchyme, thereby forming the primitive lung bud (Figure 2). The endoderm of this primitive lung bud will give rise to the epithelium and glands of the future lungs, whereas connective tissue, cartilage and smooth muscle cells are derived from the splanchnic mesenchyme surrounding these structures.[63] As lung bud outgrowth proceeds, the ventral respiratory and dorsal oesophageal parts of the foregut start to separate.

Opinions on the underlying mechanism differ. The 'traditional' theory describes lateral longitudinal tracheo-oesophageal folds that fuse in the midline in a caudal to cranial direction, to form the tracheo-oesophageal septum. Incomplete fusion of these folds would result in an abnormal connection between the trachea and the oesophagus, a tracheo-oesophageal fistula.[63]

Figure 2 Development of the trachea and the oesophagus from the primitive foregut



a. Lateral view at the end of the third week of gestation
b. en c. Ventral views of the development during the fourth week of gestation
(Reprinted with permission from: Langman J, Inleiding tot de embryologie. 9th edition, 1982, page 227. Utrecht/Antwerpen: Bohn, Scheltema & Holkema)

Others raised doubts about the existence of the longitudinal folds and proposed different theories. A few of these theories are described in the following paragraphs.[64-70]
Kluth et al. found no evidence for the 'fusing lateral ridges' in their studies in chick embryos.[65,67] Instead, they described a system of folds that grow into the tracheo-oesophageal space, but do not fuse, thereby causing tracheo-oesophageal separation. Oesophageal atresia with tracheo-oesophageal fistula could then be explained by imbalanced growth of these folds.[67,71]

In rats, Qi and Beasley described three stages of epithelial proliferation and apoptosis by which the trachea and the oesophagus separate. Disturbances in either proliferation or apoptosis would result in abnormal development and congenital anomalies.[66] The importance of apoptosis in the normal and abnormal development of the foregut was also described by Zhou et al.[72]
Studying the Adriamycin rat model, Crisera et al. came up with a new theory. Adriamycin is a glycosidic anthracyclin antibiotic used in chemotherapy. When given to pregnant rats during a specific time window in pregnancy, it produces oesophageal anomalies in the offspring. Also, other VACTERL-anomalies, as well as duodenal atresia and bladder anomalies have been described.[73] Crisera et al. observed that early atresia of the foregut was the primary event. The lung bud developed from the atretic foregut as in the normal situation. However, the lung bud did not show its usual bifurcation, but rather a trifurcation. The left and right branches developed into lungs and the middle branch grew caudally towards the stomach and ultimately connected to it, thereby forming the tracheo-oesophageal fistula.[69,70]The non-branching nature of the fistula is explained by defective epithelial-mesenchymal signalling. This model could explain the common proximal atresia with a distal fistula, but other authors could not confirm the findings.[65,68]

Orford et al., using the same model, found that the notochord in these animals is malformed and ventrally displaced and thereby located abnormally close to the developing foregut. This leads to abnormally strong signalling of Sonic hedgehog (Shh) from the notochord to the foregut. The authors propose that in the normal situation, a downregulation of Shh signalling is necessary for the separation of trachea and oesophagus. The close proximity of the notochord to the foregut in the Adriamycin rat would impede this downregulation, causing tracheo-oesophageal anomalies.[17,74] Various insults during pregnancy may initiate this faulty process, such as exposure to toxic substances and the process of twinning, possibly superimposed on an already genetically vulnerable foetus.[17]

Interestingly, abnormal notochord morphology was also observed in *Noggin* knockout mice, which show tracheo-oesophageal anomalies in 60 - 70% of cases.[75,76] *Noggin* is a BMP (Bone Morphogenetic Protein)-antagonist. Based on these knockout mice, Li et al. recently described a new possible mechanism in the development of tracheo-oesophageal anomalies. They found that the abnormal notochord detached imprecisely from the dorsal foregut endoderm, leading to a prolonged contact between notochord

and dorsal foregut. Also, presumptive dorsal foregut endodermal cells were found in the notochord, which could indicate a selective reduction of the dorsal foregut endoderm. As the dorsal foregut domain is made up of only a few cells, this may then lead to atresia of the oesophagus.[76]

In summary, although many different mechanisms have been proposed in the literature, we cannot but conclude that the exact development of tracheo-oesophageal anomalies remains unclear.

Aetiology

The aetiology of tracheo-oesophageal anomalies is largely unknown and is considered to be multifactorial, with roles for both genetic and environmental factors. However, the exact nature and interplay of these factors is as yet unknown. Genetic factors may predominate in some cases, with no or minor roles for environmental factors. Alternatively, one or more specific environmental factors may be responsible, especially if a foetus is already genetically predisposed, and thus more vulnerable to teratogenic insult. Several authors described that tracheo-oesophageal anomalies may occur more frequently in the offspring of younger mothers.[8,77,78] Others, however, have not been able to confirm this association.[9,10]

Environmental factors

Over the years, various environmental factors have been suggested to play a role in the development of tracheo-oesophageal anomalies, such as exposure to certain medications,[79-83] alcohol,[84,85] or infectious diseases.[86,87] A possible association with exogenous sex hormones has been suggested as well.[88-94] Several other studies, however, could not confirm this association.[95-102] So far, no specific environmental risk factor has consistently been identified.[77,103-106]

Vitamin A deficiency

In the 1940s, experiments were performed in which pregnant rats were fed a vitamin A deficient diet. The offspring of these rats displayed many severe congenital anomalies, mainly of the eyes, the urogenital system and the diaphragm, but tracheo-oesophageal fistula was also found.[107] Later studies in transgenic mice with compound null mutations of different isoforms of the Retinoic Acid Receptors also revealed defects of tracheo-oesophageal separation. It would seem, therefore, that retinoic acid, the active derivative of vitamin A, is indispensable for normal embryologic development.[108] There have been no reports of vitamin A deficiency in human mothers of affected children.

Genetic factors

OA/TOF may occur as an isolated anomaly, may be part of a complex of congenital defects, or may develop within the context of a known syndrome or association. Genetic factors are most likely to play a role in the latter two groups. Table 2 lists syndromes and

associations in which tracheo-oesophageal anomalies have been described. The most relevant ones will be discussed in more detail in chapter 3 of this thesis.

Although familial cases of oesophageal atresia are rare, there are incidental reports of two or three affected siblings and of concordant or discordant twins; in general, however, the concordance rate is low.[10,13,78,109-117] The estimated recurrence risks are 0.8 - 1.7% and 20%, for the second and third child, respectively.[8,118]
Recurrence in the next generation has also been reported.[78,114-116,119] A parent with OA/TOF has a 2 - 4% chance of having a child with the same anomaly.[118,120]

The rare familial occurrence might suggest that genetic factors do not play a major role in the aetiology. Nevertheless, there is growing evidence that specific genetic anomalies may cause tracheo-oesophageal anomalies in certain instances, as is described in chapter 3 of this thesis.

Chromosomal anomalies
Chromosomal anomalies have been reported in 6 - 10% of patients with OA/TOF. Trisomies 21 and 18 predominate.[8-10,121] Over 30 reports describe specific chromosomal anomalies, and these are further discussed in chapter 3 of this thesis. No single chromosomal defect has been described as a proven aetiological factor. However, these chromosomal anomalies can serve as a starting point in the search for candidate regions and genes involved in the development of tracheo-oesophageal anomalies.

Knockout mice
From transgenic mice studies, a number of genes have been implicated in the development of OA/TOF (for an overview see chapter 2 of this thesis). Finding a phenotype of OA/TOF in mice lacking function of a specific gene does not necessarily imply a causal role for this gene in the human pathogenesis. These studies should be complemented by functional studies, such as expression experiments (e.g. immunohistochemistry or RT-PCR) in both humans and animals. A number of genes identified in knockout experiments have been studied in animal and human tracheo-oesophageal fistula samples, as described below.

Adriamycin model
The Adriamycin rat model, which was described earlier in this chapter, has been used in many studies on the embryology of tracheo-oesophageal anomalies, as well as on histological, immunohistochemical and genetic features these anomalies.[17,122-125] However, no association between Adriamycin or structurally related substances and tracheo-oesophageal anomalies has been reported in humans.[126-128]

Morphology of the tracheo-oesophageal fistula
Histological studies of the tracheo-oesophageal fistula and distal oesophagus show a mixed picture. Animal studies have demonstrated respiratory-like pseudostratified

columnar or ciliated epithelium and cartilage.[69,129] Merei et al. described respiratory-like epithelium, which changed into stratified squamous epithelium at variable distances from the cranial origin of the TOF.[130,131] Human samples have not only shown (pseudo-)stratified squamous epithelium, but also a more respiratory-type structure, with tracheobronchial remnants (such as cartilage) and ciliated epithelium. In addition, abnormal mucous glands and a disorganised muscular coat (as opposed to the well-organised muscular layers of the normal oesophagus) have been described.[132-135]

Expression studies in the tracheo-oesophageal fistula

By means of immunohistochemistry, RT-PCR and *in situ* hybridisation, the expression of specific proteins and genes has been studied in both animals and humans.

TTF-1

In Adriamycin rat embryos, Thyroid Transcription Factor 1 (TTF-1) expression was found in normal trachea and lungs, but not in normal oesophagus. Expression of TTF-1 was also found in tracheo-oesophageal fistulas, becoming very weak in late gestation. The proximal oesophageal pouch did not show any TTF-1 expression.[122,136]

Spilde et al. confirmed the presence of TTF-1 in human fistulas, albeit weak and in only two specimens.[137]

Adapting the Adriamycin model to the mouse, Ioannides et al. found that TTF-1 was either not expressed at all in the fistula tract, or only at its origin in the ventral part, disappearing after a short distance. While TTF-1 was not found in the oesophagus, strong expression was described in respiratory structures.[138] Differences between species might explain the different results found.

Shh

Spilde et al. showed that Sonic hedgehog (Shh) is absent from the human fistula, but present in the muscular layers of the proximal oesophagus in one patient.[134]

The above data for TTF-1 and Shh seem to support a respiratory origin of the fistula, but lack strength because no more than two and one human fistulas, respectively, were examined.[122,134,136,137]

If the fistula is indeed a third branch arising from the tracheal bifurcation, as suggested by Spilde et al. and Crisera et al., its non-branching nature in contrast to both main bronchi may be explained by defective ligand-receptor signalling in the epithelium or the mesenchyme.[69,70] Bone Morphogenetic Proteins (BMPs) are known to be involved in normal lung branching and cellular proliferation and differentiation, and BMP signalling may be implicated in the development of tracheo-oesophageal fistulas.[139]

BMPs and BMP receptors

Crowley et al. studied the expression patterns of BMP 2, 4 and 7 and BMP-receptors (BMPR) IA, IB and II in the Adriamycin rat model as well as in human samples.[139,140] Table 3 provides an overview of the findings from these studies.

In summary, in the Adriamycin model, the fistula showed a respiratory-like expression pattern in early gestation, later changing to a more oesophagus-like pattern. However, as expression patterns in normal trachea and in the proximal pouch were not described, it is hard to draw definite conclusions.[140] In human samples, the fistula tract showed a mixed pattern, with BMPs being absent (comparable to trachea) and BMP-receptors also absent, except for BMPRII (comparable to oesophagus). Expression patterns in the proximal pouch were identical to those in normal oesophagus. The authors propose that a disruption of BMP signalling in the fistula tract would explain the non-branching nature of the tracheo-oesophageal fistula, which they consider to originate from a trifurcation of the embryonic lung bud.[139] 60 - 70% of mice deficient for *Nog*, a BMP-4 antagonist, were found to have OA/TOF, but *NOG* mutations have not been found in humans with OA/TOF.[75,76]

The theory of disrupted signalling as a cause for the non-branching of the TOF is supported by earlier papers showing defective fibroblast growth factor signalling.[123,125,141,142]

Tabel 3 Expression of BMPs and BMP receptors as described by Crowley et al. (see text for references)

	Adriamycin rat model					**Human samples**				
	Normal lung	Normal trachea	Normal oesophagus	TOF	Proximal oesophageal pouch	Normal lung	Normal trachea	Normal oesophagus	TOF	Proximal oesophageal pouch
BMP 2	**-**	N/r	**+**	Early - Late +	N/r	**+**	**-**	**+**	**-**	**+**
BMP 4	-	N/r	+	Early - Late +	N/r	+	-	+	-	+
BMP 7	-	N/r	+	Early - Late +	N/r	+	-	+	-	+
BMPR IA	-	N/r	Early - Late +	Early - Late +	N/r	+	-	-	-	-
BMPR IB	-	N/r	Early - Late +	Early - Late +	N/r	+	+	-	-	-
BMPR II	Early - Late +	N/r	Early - Late +	Early - Late +	N/r	+	-	+	+	+

BMP: Bone Morphogenetic Protein; BMPR: Bone Morphogenetic Protein Receptor; N/r: not reported; Early: early gestation; Late: late gestation; TOF: tracheo-oesophageal fistula

OUTLINE OF THE THESIS

As mentioned before, not much is known about the possible causes of tracheo-oesophageal anomalies. More can be learned by systematically collecting and studying relevant information in databases such as the one in existence at the Erasmus MC-Sophia Children's Hospital. In this database, data on all children with OA/TOF treated in the Children's Hospital are stored. Records contain patient characteristics, information on pregnancy, delivery and birth, family history and associated malformations.
It is also still unknown whether genetic factors or environmental factors predominate in the aetiology of OA/TOF. It is likely, that the contributions of these factors differ between (groups of) cases. To gain more insight in the aetiology of OA/TOF, a number of studies into both genetic and environmental factors have been conducted at the Sophia Children's Hospital in recent years, and these are described in this thesis.

Part I gives an overview of the subject. **Chapter 1** gives an introduction to OA/TOF. A review of relevant candidate genes identified in transgenic animal models is provided in **chapter 2** and associated chromosomal anomalies in patients reported in the literature are described in **chapter 3**.

Part II describes some phenotypical aspects of OA/TOF. **Chapter 4** is an epidemiological study into associated anomalies in children with OA/TOF and the most frequently occurring association, the VACTERL association. In **chapter 5**, a relatively large group of patients with tracheal agenesis, a rare, but related congenital anomaly of the foregut, is described.

Part III describes studies into molecular aspects of the aetiology. **Chapter 6** shows an analysis of gene expression patterns in tracheo-oesophageal fistulas using gene expression arrays. A patient with a mutation in *MYCN*, a specific gene involved in syndromic OA, is described in **chapter 7**.

Part IV describes studies into environmental factors. **Chapter 8** is an analysis of questionnaires asking about factors during pregnancy. This study was performed in collaboration with the Dutch parents' association of children with OA/TOF (VOKS or Vereniging voor Ouderen en Kinderen met een Slokdarmafsluiting). **Chapter 9** shows a possible association between maternal *in utero* exposure to a synthetic oestrogen and OA/TOF in their children.

In **Part V**, the results of this thesis are discussed (**chapter 10**) and summarised (**chapter 11**).

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Genetics and developmental biology of oesophageal atresia and tracheo-oesophageal fistula: lessons from mice relevant for paediatric surgeons

Chapter 2

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ABSTRACT

Oesophageal atresia and tracheo-oesophageal fistula are relatively frequently occurring foregut malformations of which the aetiology and pathogenesis are poorly understood. Recent results of molecular genetic studies, in particular the use of single and compound mutant mice, have yielded a tremendous increase in the understanding of the molecular mechanisms involved in normal and abnormal foregut morphogenesis. In the introduction of this paper, we review the very early stages of normal and abnormal embryology of the foregut derivatives and the separation of the foregut into a ventral respiratory part and a dorsal digestive part. After that, we describe the genes that have been demonstrated to play important roles in these processes.

INTRODUCTION

Oesophageal atresia (OA) and tracheo-oesophageal fistula (TOF), a persistent connection between the trachea and the oesophagus, are among the most frequently occurring major anatomical anomalies of the foregut and are well-known disease entities in paediatric surgery. TOF and/or OA occur in approximately one in 3,500 newborns, and their aetiology is poorly understood.[1] No specific chromosomal anomalies related to the aetiology of OA and TOF have been described, apart from one study concerning OA as part of familial oculodigitoesophagoduodenal (ODED) syndrome.[2]

It is suggested that TOF results from an incomplete division of the ventral and dorsal parts of the foregut, which constitute the respiratory and digestive parts, respectively.[3] Five types of oesophageal atresia are recognised. The most common variant is proximal atresia with a distal TOF, occurring in 85% of cases.[4] Other fairly rare anomalies of the foregut are tracheal diverticulum and tracheal stenosis and atresia. The latter two anomalies are usually associated with TOF.

In recent years, the embryology of the foregut in humans has been the subject of much controversy, and no agreement exists about the exact mechanisms of (ab)normal development. In humans, normal early development of the foregut begins during the 4th week of embryonic life, with the differentiation of the foregut endoderm into a ventral respiratory part and a dorsal oesophageal part. From the ventral side, the laryngotracheal diverticulum evaginates from the foregut and grows into the splanchnic mesenchyme, thereby forming the primitive lung bud. The endoderm of this primitive lung bud will give rise to the epithelium and glands of the future lungs, whereas connective tissue, cartilage, and smooth muscle cells are derived from the splanchnic mesenchyme surrounding it.

In the "traditional" theory, separation of the ventral respiratory part from the dorsal oesophageal part is achieved by the formation of lateral longitudinal tracheo-oesophageal folds, which fuse in the midline to form the tracheo-oesophageal septum. Six to seven weeks after ovulation, the separation of the oesophagus and the trachea is complete.[3] Incomplete fusion of the tracheo-oesophageal folds will result in the formation of a defective tracheo-oesophageal septum and thus an abnormal connection between the trachea and the oesophagus.[3]

Over the years, there has been controversy about the existence of the tracheo-oesophageal septum and of the longitudinal folds.[5-8] A number of theories about the pathogenesis of tracheo-oesophageal anomalies have been put forward.[6,9]

Kluth et al. found no evidence for the appearance of these fusing lateral ridges in their studies in chick embryos.[6,8] Instead, they described a system of cranial and caudal folds in the area of tracheo-oesophageal separation. According to this theory, oesophageal

atresia with tracheo-oesophageal fistula can be explained by imbalances in the growth of these folds.[8,10]

Qi and Beasley, studying a rat model, divided the process of separation of the trachea and the oesophagus into three stages of epithelial proliferation and apoptosis. Due to disturbances in either proliferation or apoptosis, different forms of tracheo-oesophageal anomalies occur.[7]
Although many different models have been proposed in the literature, the exact mechanism of development of tracheo-oesophageal anomalies remains unclear.

The Adriamycin rat model of oesophageal atresia has greatly enhanced our knowledge of the pathogenesis of OA and TOF.[11] With this model, OA-TOF can be studied in the early embryonic period. From observations made in these rats, Crisera et al. described a new concept of the development of TOF. The embryos in this model developed early atresia of the foregut. The lung bud developed from the atretic foregut as described in the normal foregut. However, the lung bud did not show its usual bifurcation, but rather a trifurcation. The left and right branches developed into lungs, and the middle branch grew caudally towards the stomach and ultimately connected to it, thereby forming the TOF.[12] This model could explain the most common type of OA, proximal atresia with a distal fistula. However, it has not been confirmed by other authors.[6,9]

Although interesting from an embryological point of view, these animal data provide no information about genes involved in early foregut development. Knockout studies in mice have recently elucidated the functions of different genes involved in foregut development in general and tracheo-oesophageal separation in particular. In this paper, we present a short overview of the molecular pathways of the genes involved, based on studies in single and compound null mutant mice as well as some preliminary but promising data in humans. We focus specifically on genes that were shown to play an essential role in the separation of the primitive foregut into a ventral and a dorsal part.

GENETICS OF OESOPHAGEAL ATRESIA AND TRACHEO-OESOPHAGEAL FISTULA (Table)

RAR

Since the early 1950s, the essential role of vitamin A in embryogenesis has been established. Early experiments showed the effects of dietary vitamin A deficiency (VAD) in rats during development that result in VAD syndrome, which includes malformations of the eyes, respiratory tract, cardiovascular system, urogenital system, and diaphragm.[13]
Vitamin A exerts its effects through two families of receptors, the retinoic acid receptors (RAR) α, β, and γ, which all three have a number of isoforms, and the retinoid X receptors (RXR) α, β, and γ.

Compound null mutations of *RAR* and *RXR* genes demonstrate that they are essential for vertebrate ontogenesis.[14,15] More specifically, in *RARα*$^{-/-}$; *β*$_2^{-/-}$, *RARα*$_1^{-/-}$; *β*$^{-/-}$ and *RXRα*$^{-/-}$; *RARα*$^{-/-}$ double-mutant mice, the tracheo-oesophageal septum is absent. In addition, these mice exhibit lung hypoplasia or even lung agenesis.[16,17] This phenotype is highly similar to phenotypes observed in VAD rat foetuses and thus corroborates with the concept of vitamin A being essential for foregut morphogenesis. Retinoic acid is involved in the earliest *Hox* gene expression.[18,19] *RARα* has been mapped to 17q21.1, *RARβ* to 3p24 and *RXRα* to 9q34.3 in humans.[20,21,22]

Sonic hedgehog

A striking example of the highly conserved function of certain proteins throughout evolution is given by the hedgehog proteins, first described in the fruitfly Drosophila melanogaster in 1980.[23] Mammalian homologues were soon discovered and were shown to be key regulators of morphogenesis in vertebrates (for review, see ref. 24-26). The hedgehog proteins, given names like Sonic, Indian, and Desert, are important signalling molecules that all have their own expression patterns.[24]

Sonic hedgehog (shh) knockout mice display abnormal foregut morphogenesis resulting in oesophageal atresia, failed separation of the trachea and oesophagus, and lungs that form bilateral rudimentary sacs that arise from a single tracheo-oesophageal tube.[27] In addition, they show nervous system defects.[28] This phenotype is remarkably similar to the phenotype described in null mutant mice for *Ttf-1*[29] (see paragraph on Ttf-1 for further details). However, the expression of Ttf-1 in *shh*$^{-/-}$ lungs and that of shh in *Ttf-1*$^{-/-}$ lungs was not altered, indicating that Shh and Ttf-1 function through independent pathways despite the similar phenotype of homozygous inactivation of both genes.[29,30] *Sonic hedgehog* has been located to 7q36 in humans.[31]

Gli family

In mammals, three zinc-finger transcription factors named Gli1, Gli2, and Gli3 are downstream targets of *Shh*.[25] Null mutant mice for *Gli1* are viable and do not have apparent abnormalities. Heterozygosity for *Gli2* in *Gli1* knockout mice or homozygosity for both *Gli1* and *Gli2* null mutations causes multiple defects, including anomalies of the respiratory tract, but OA or TOF have not been described.[32] Homozygous inactivation of Gli2 has been reported to result in tracheo-oesophageal stenosis and also hypoplasia and lobulation defects of the lung. When *Gli3* gene dosage is reduced by 50% in this *Gli2*$^{-/-}$ background, the result is OA with TOF and a more severe lung phenotype.[33] Double mutant mice for both *Gli2* and *Gli3* do not form the foregut derivatives oesophagus, trachea, and lung.[33] Taken together, these results indicate an essential role for Gli2 and Gli3 in foregut morphogenesis by modulation of Shh signalling.

In humans, mutations in the *Gli3* gene have been implicated in a number of genetic disorders, namely Greig cephalopolysyndactyly syndrome, Pallister-Hall syndrome (PHS), and the polydactyly syndromes postaxial polydactyly type A and type A/B and autosomal

dominant preaxial polydactyly type IV syndrome (for review, see ref. 25). In PHS patients, foregut malformations such as pulmonary segmentation defects, tracheal stenosis, and TOF have been observed.[34,35] *Gli2* and *Gli3* have been mapped to 2q14 and 7p13, respectively, in humans.[36,37]

Foxf1

The forkhead family is a family of transcription factors that share a common DNA-binding domain.[38] Members of the forkhead family of transcription factors have been shown to be involved in the formation and differentiation of tissues derived from the foregut endoderm.[39,40]

Foxf1, previously known as Hfh8 or FREAC1, is a member of the forkhead family that is involved in foregut development. *Foxf1*-null mutant mice die in utero before embryonic day 10, due to extra-embryonic mesoderm defects. A study by Mahlapuu et al. showed that heterozygous mice have a high perinatal mortality and exhibit multiple defects in foregut-derived structures, including lung hypoplasia, lung immaturity, and lobulation defects as well as OA and TOF.[40] Kalinichenko et al. described fatal pulmonary haemorrhage in a subset of *Foxf1*$^{+/-}$ mice. However, the morphology of the oesophagus is not discussed in this paper.[41]

Similar anomalies were also demonstrated in mice lacking *sonic hedgehog* and it has been suggested that Foxf1 is involved in the Sonic Hedgehog pathway, downstream of Shh.[40] The human *Foxf1* gene has been localised to chromosome 16q24.[42]

Ttf-1

Thyroid transcription factor-1 (Ttf-1), also known as Nkx2.1 or thyroid-specific enhancer binding protein, is a homeodomain protein that is involved in regulating transcription of certain thyroid- and lung epithelium-specific genes.[43,44] It upregulates expression of genes in the cells of the terminal airway epithelium that produce surfactant.[43,45,46]

Null mutation of *Ttf-1* results in failed separation of the trachea and the oesophagus. *Ttf-1*$^{-/-}$ mice exhibit a severe lung phenotype, including rudimentary peripheral lung primordia arising from a single tracheo-oesophageal lumen, resulting in perinatal lethality. In addition, these mice do not have a thyroid gland and show severe abnormalities in the brain, including an absence of the pituitary.[29,47] Expression of Ttf-1 is stimulated by Foxa2[48] and Gata-6,[49] which will be briefly discussed in the last part of this paper.

In humans, a functional deletion of the *Ttf-1* gene was described in an infant with neonatal thyroid dysfunction and respiratory failure.[50] This confirms that the function of Ttf-1 during lung development is conserved in both humans and mice. The gene locus for *Ttf-1* in humans is on 14q13.[51]

Nog

Noggin (Nog) is a Bone Morphogenetic Protein 4 (Bmp4) antagonist. BMPs are known to be involved in normal lung branching and cellular proliferation and differentiation.[52] Animal studies have shown that Noggin is involved in foregut development. Around 60% of null mutants for *Nog* display OA/TOF, in addition to very abnormal lung branching.[53] *Nog* is located on chromosome 17q22 in humans.[54] However, mutations in *Nog* have not been associated with OA/TOF in humans.[53]

Hoxc4

The clustered *homeobox (Hox)* genes are a large family of genes that are expressed in a segmental pattern and play important roles in morphogenesis in many species, encoding a highly conserved family of transcription factors.[18]

Hoxc4 is expressed in low quantities in the endoderm of the developing lung and trachea and also in oesophageal endoderm and mesoderm. Null mutant mice for *Hoxc4* exhibit a partially or completely blocked oesophageal lumen and a disruption of oesophageal musculature. In addition, they have anomalies of the thoracic vertebrae.[55] The blockage of the oesophageal lumen seems to be due to an increase in proliferation of the epithelium, suggesting that Hoxc4 might have a limiting role in cell proliferation of the oesophageal epithelium.[55]

Null mutant mice for *Hoxc4* also show a significant reduction in the expression of Hoxc5. This reduction may play a role in the knockout phenotype, although *Hoxc5*-null mutants show no oesophageal anomalies.[55] The gene locus for *Hoxc4* in humans is on 12q13.3.[56]

Tbx4

The *T-box (Tbx)* genes are a family of transcription factors that play important roles in the development of different species.[57-59]

Tbx4 is expressed in the lung bud and trachea in chicken embryos[58] and is speculated to be involved in the separation of trachea and oesophagus. Transient misexpression of Tbx4 in the prospective oesophageal-respiratory region rather than null mutation results in failure of formation of the tracheo-oesophageal septum.[60] The human gene locus for *Tbx4* is on chromosome 17q21-q22.[61]

Table Genes involved in normal tracheo-oesophageal development

Gene	Mutant phenotype	Human locus
RARα$^{-/-}$;β$_2^{-/-}$ or RARα$_1^{-/-}$;β$^{-/-}$ or RXRα$^{-/-}$; RARα$^{-/-}$	Tracheo-oesophageal fistula Lung hypoplasia or agenesis	RARα: 17q21.1 RARβ: 3p24 RXRα: 9q34.4
Shh$^{-/-}$	Oesophageal atresia Tracheo-oesophageal fistula Lungs form rudimentary sacs	7q36
Gli2$^{-/-}$;Gli3$^{+/-}$	Oesophageal atresia Tracheo-oesophageal fistula Severe lung phenotype	Gli2: 2q14 Gli3: 7p13
Gli2$^{-/-}$;Gli3$^{-/-}$	No formation of oesophagus, trachea and lungs	
Foxf1$^{-/-}$	Lethal before embryonic day 10 Extra-embryonic defects	16q24
Foxf1$^{+/-}$	Oesophageal atresia Tracheo-oesophageal fistula Lung immaturity/hypoplasia Lobulation defects	
Ttf-1$^{-/-}$	Tracheo-oesophageal fistula Rudimentary peripheral lung primordia	14q13
Noggin$^{-/-}$	Oesophageal atresia Tracheo-oesophageal fistula Lung branching defects Abnormal notochord morphogenesis	17q22
Hoxc4$^{-/-}$	Partially or completely blocked oesophageal lumen Disruption of oesophageal musculature	12q13.3
Tbx4 misexpression	Tracheo-oesophageal fistula	17q21-q22

DISCUSSION

The embryological development of the foregut into a respiratory and a digestive part is a complicated process, of which many aspects have not yet been clarified. It is thought that a combination of genetic and environmental factors plays a role in the aetiology of foregut anomalies. In this paper, we reviewed the current status of the genes that have been demonstrated to be essential for normal tracheo-oesophageal separation and development. The phenotypes of null mutant mice for these genes displayed abnormal foregut development resulting in OA and/or TOF, with the exception of Tbx4, in which misexpression rather than null mutation plays a role.

Besides the genes discussed in this paper, many others are involved in foregut development in general. An in-depth discussion of the exact function of these genes is outside the scope of this paper. However, a brief mention of a few of them is given here.
The **GATA family of transcription factors** is involved in the determination of foregut endoderm. Null mutant mice for either *GATA-4* or *GATA-6* exhibit severe defects in foregut morphogenesis.[62-65]

Foxa2, a member of the forkhead family that was originally described as a liver-specific gene and previously known as hepatocyte nuclear factor-3β (HNF-3β), has been shown to play an important role in the specification of foregut endoderm and the maintenance of definitive endoderm (for review, see ref. 66).

IKKα (IκB kinase-α) and RIP4 (receptor interacting protein 4) are genes that play a role in keratinocyte differentiation.[67,68] Null mutant mice for either of these genes show a thickened, undifferentiated epidermis with an increased adhesiveness. Among other more generalised defects, this causes fusion of the gut epithelium, which leads to blockage of the oesophageal lumen.[67,68]

The origin of TOF has been the subject of a number of studies in recent years.[69-71] In rats with Adriamycin- induced OA, Ttf-1 was shown to be expressed in the fistula, the distal oesophagus and the lungs, but not in the proximal oesophagus or the stomach, suggesting a respiratory origin of the fistula.[69] In addition, the fistula branched in response to exogenous Shh, as did normal lung but not normal oesophagus, supporting the theory of a respiratory origin.[71]

Spilde et al. confirmed the presence of Ttf-1 in human fistulas.[70] They also showed that Shh is absent from the distal fistula but present in the proximal oesophagus, providing support for a respiratory origin of the fistula in humans.[72] However, only two fistulas were examined. More research into gene expression patterns in human fistulas is needed to determine the exact origin of the fistula.

During the past decade, major progress has been made in understanding the genetic mechanisms during embryogenesis in general and foregut development in particular. This is mainly based on candidate-gene approaches using knockout mice in which the effects of homozygous inactivation of certain genes are investigated. From these studies we have gained more insight into the molecular mechanisms involved. Moreover, they have demonstrated that these mechanisms are highly conserved in vertebrate foregut morphogenesis. The demonstration of gene mutations in *TTF-1* and *Gli3* in human patients with similar phenotypes showed that mice knockout studies may provide us with essential information about the genes involved in foregut anomalies.

With the expansion of new (faster) molecular biotechnologies (such as biochips) and bioinformatics, a new era awaits us in which the genes involved in abnormal (foregut)

embryogenesis will be elucidated rapidly. The first challenge for the future will be to link phenotypes derived from mice gene knockout studies to phenotypes of human patients, which will give information about the roles of genes involved in abnormal embryology leading to human anomalies. Consequently, phenotypic description of each individual patient and consultation by a clinical geneticist is warranted. Moreover, the development of routine karyotyping together with the availability of DNA and tissue banks of patients with congenital anomalies for molecular genetic evaluation will lead to increased knowledge of the aetiology of these anomalies and will be of value for future research in this area. The collection of karyotypes and DNA samples may serve as a standard approach for patients with major congenital anomalies who come to the attention of the paediatric surgeon.

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Chromosomal anomalies in the aetiology of oesophageal atresia and tracheo-oesophageal fistula

Chapter 3

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ABSTRACT

Oesophageal atresia (OA) and tracheo-oesophageal fistula (TOF) are severe congenital anomalies of which the aetiology is largely unknown. Several chromosomal anomalies have been described in patients presenting with these anatomical malformations, but until now none of these has led to the identification of a single aetiological factor. This paper reviews the chromosomal abnormalities reported in cases of OA/ TOF and serves as a starting point to identify chromosomal regions harbouring genes involved in the aetiology of OA/TOF.

INTRODUCTION

Oesophageal atresia (OA) and tracheo-oesophageal fistula (TOF) are relatively frequently occurring malformations of the foregut, with a prevalence of around 1 in 3,500 births. Associated anomalies occur in some 50% of cases.[1] The best known association of anomalies is the VACTERL (Vertebral, Anal, Cardiac, Tracheo-oEsophageal, Renal, Limb) association, found in around 10% of infants with OA/TOF.[2]

The aetiology of these anomalies is as yet poorly understood, but it is generally considered to be multifactorial. Environmental factors, such as exposure to certain medications[3,4] or infectious diseases[5] might be risk factors for isolated OA or VACTERL association, but no external factor has consistently been identified.[6-8] Chromosomal anomalies have been reported in 6 - 10% of patients with OA/TOF and are mostly trisomies.[9,10] No single specific chromosomal defect has been described as a proven aetiological factor. Recently however, four genes, *MYCN, CHD7, SOX2* and *MID1*, were reported to be involved in the aetiology of syndromic OA/TOF (for recent reviews: Brunner & Van Bokhoven and Shaw-Smith).[11,12]

Knockout studies in mice implicate genes like *Sonic hedgehog (Shh), Gli2, Gli3, TTF-1* and *RARα* and *β* in the development of OA/TOF (for review: Felix et al., 2004).[13] As described below, deletions in or close to the regions of *SHH* (7q36) and *RARα* (17q21.1) have been reported in humans with OA/TOF. Nevertheless, the chromosomal defects in these patients have not been proven to be causes of the oesophageal anomalies.

Over 30 reports describing chromosomal anomalies in children with OA/TOF have been published. These reports could serve as a starting point in the search for genes involved in the development of OA/TOF.

This paper presents an overview of the findings from these reports in combination with data from our hospital-based database of cases with OA/TOF.

HOSPITAL-DATABASE

Clinical characteristics, including karyotypes when available, of all 255 patients with OA/TOF treated in the Erasmus MC-Sophia Children's Hospital in Rotterdam, the Netherlands, since 1988 have been stored in a database. This hospital is the paediatric referral center for the South western part of the Netherlands, with a population of around four million people and approximately 35,000 births per year. It has the only specialized paediatric surgical intensive care unit in the Netherlands. Chromosomal anomalies found in patients in the database are shown in Table 1. Karyotypes are known for 53 of 105 patients (50.5%) born between 1988 and 1995 inclusive, 50 of 71 patients (70.4%) born between 1996 and 2000 inclusive, and 66 of 79 patients (83.5%) born between 2001 and now.

Table 1 Chromosomal anomalies found in children with OA/TOF treated in the Erasmus MC-Sophia

Chromosomal anomaly	Number of patients	Remarks
47,XX/XY,+21	6 (1 mosaic)	3 girls, 3 boys
47,XX,+18	1	
47,XY,+13	1	
47,XXX	1	
46,XY,t(5; 10)(q13;q23)pat	1	Father no phenotype
46,XX; 46,XY	160	64 girls, 96 boys
Unknown	85	26 girls, 59 boys

The proportion of cases of trisomy 21 linked with OA and/or TOF in our hospital (2.4%) is comparable to those found in the literature.[1,8] However, we documented only one case of trisomy 18, which does not compare with findings from other studies: Torfs et al. report 7.2% and Robert et al. 3.2%.[1,8] The pregnancies of children with trisomy 18 were quite likely terminated and therefore the children would not have been seen in our department. The numbers in the other groups are too small to draw conclusions from.

CHROMOSOMAL ANOMALIES DESCRIBED IN ASSOCIATION WITH OA AND/OR TOF

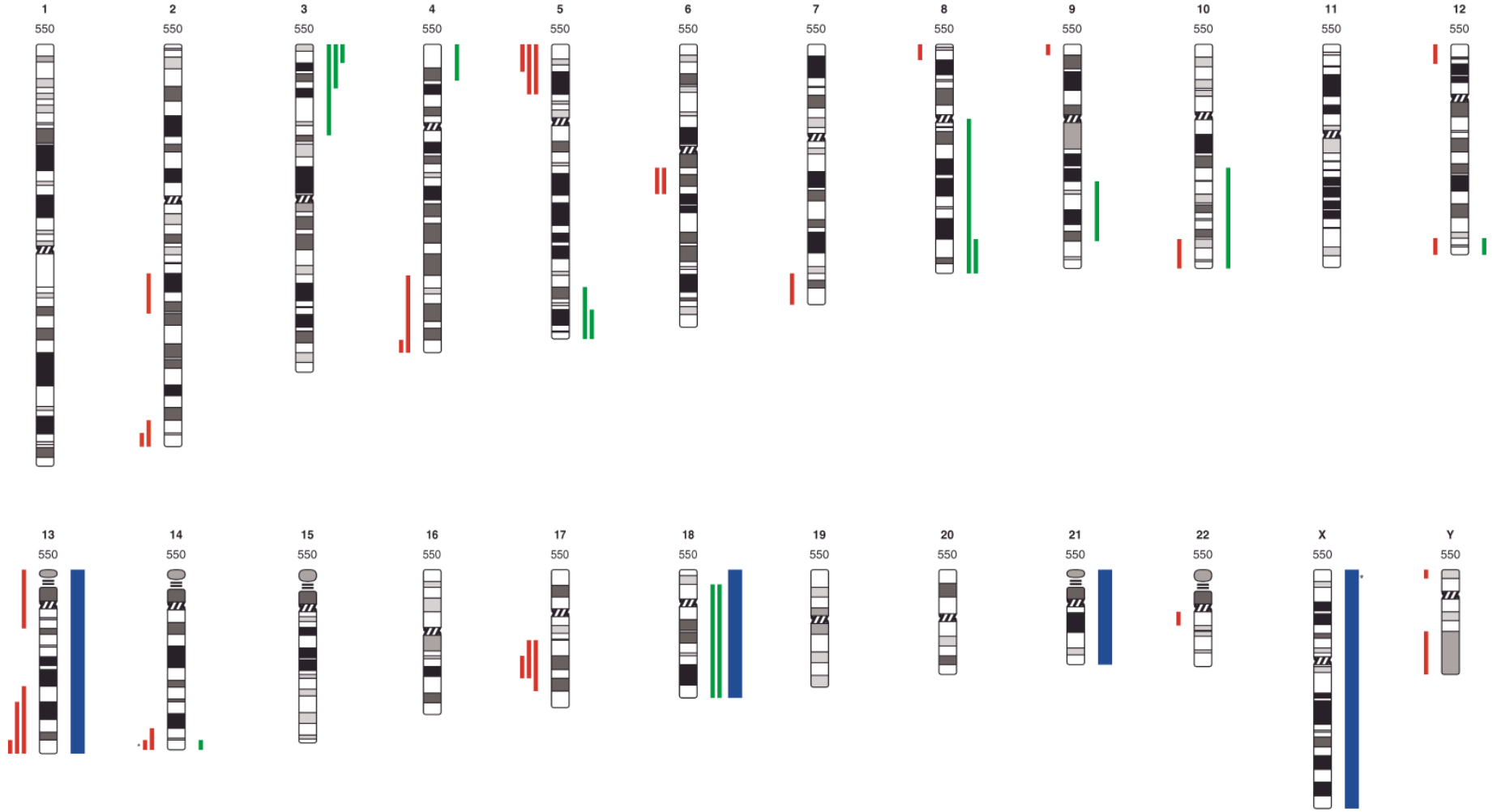
The PubMed electronic database of the U.S. National Library of Medicine was searched using all possible combinations of: (o)esophageal atresia or tracheo(-)(o)esophageal fistula combined with: chromosome(s), chromosomal anomaly or anomalies and gene(s). In addition, the first two terms were combined with the word "chromosome" followed by numbers 1 to 22, X and Y (e.g. oesophageal atresia and chromosome 1, oesophageal atresia and chromosome 2, etc.).

There were no restrictions to year of publication. All references of papers found through this search were also reviewed to identify any studies that may have been missed.

For every chromosomal region described in association with OA/TOF, the corresponding sections of A. Schinzel's "Catalogue of unbalanced chromosome aberrations in man" were studied to find reports of cases with OA/TOF showing the same chromosomal defect.[14]

Several chromosomal anomalies have been reported in the literature in cases with OA/TOF. All are summarised in Table 2 and Figure 1. Those seen in 3 cases or more are described below.

Figure 1 Chromosomal anomalies described in patients with OA/TOF (*for colour figure see page 220*)



*Deleted regions are depicted in red to the left of the chromosome, duplicated regions in green to the right of the chromosome. Blue bands to the right of the chromosome denote trisomies. Single cases are depicted as single narrow bands. Three or more cases with the same anomaly are shown as broad bands. Triploidy (2 cases), translocations and cases with unspecified breakpoints are not shown. MYCN, SOX2, CHD7 and MID1 denote specific genes in which deletions and/or mutations have been described in patients with OA/TOF (for review see ref. 11 and 12). See text for details. *Mosaic in 1 case.*

Table 2 Chromosomal anomalies found in patients with oesophageal atresia and/or tracheo-oesophageal fistula

Chromosome	Deleted segment	Duplicated segment	Karyotype	References
Chromosome 2	2q22q24.2		46,XX,del(2)(q22q24.2)	15
	2q37.1qter	14q32.3qter	46,XX,der(2)t(2;14)(q37.1;q32.3)mat	16
	2q37.2qter	12q24.13qter	46,XY,der(2),t(2;12)(q37.2;q24.13)pat	17
Chromosome 3		3p21pter	46,XY,der(7)ins(7;3)(q31p26p21)	18
	5p14pter	3p23pter	46,XX,der(5)t(3;5)(p23;p14)mat	19
	4q35qter	3p25pter	46,XY,der(4)t(3;4)(p25;q35)mat	20
Chromosome 4	4p		46[a],4p-[b]	21
		4p15.32pter 18p11.21qter	47,XX,+der(18)t(4;18)(p15.32;p11.21)	22
	4q31qter		46,XY,del(4)(q31)	23
	4q35qter	3p25pter	46,XY,der(4)t(3;4)(p25;q35)mat	20
Chromosome 5	5p14pter		46,XY,del(5)(p14)	24
	5p14pter	3p23pter	46,XX,der(5)t(3;5)(p23;p14)mat	19
	5p15pter	5q32qter	46,XY,rec(5)dup(5q)inv(5)(p15q32)pat	25
	12p13.1pter	5q34qter	46,XY,der(11)t(11;12)(p15.3;q12),der(12) t(5;12;11;12)(q34;q12;p15.3;p13.1)	26
Chromosome 6	6q13q15		46,XX,del(6)(q13q15)	27
	6q13q15		46,XY,rec(6)del(6q)ins(6)(p21.3;q15q13)mat	28
			46, XX, t(6;8)(6p8p;6q8q)	29
			46[a],t(6;9)[b]	8
			46, XX, t(6;15)(p11.2;p12)	30
Chromosome 7	7q34qter	8q24.1qter	46,XX,der(7)t(7;8)(q34;q24.1)	31
Chromosome 8	8p23pter		46,XY,del(8)(p23)	32
	13q34qter	8q11qter	46,XY,der(13),t(8;13)(q11;q34)mat	33
	7q34qter	8q24.1qter	46,XX,der(7)t(7;8)(q34;q24.1)	31
			46, XX, t(6;8)(6p8p;6q8q)	29

Chromosome 9	9p24pter 9q22.1q33	10q22qter 9q22.1q33	46,XY,der(9)t(9;10)(p24;q22) 46,XX,der(15)ins(15;9)(q21;q22.1q33)mat 46[a],t(6;9)[b]	34 35 8
Chromosome 10	10q25.3qter 9p24pter	 10q22qter	46,XX,del(10)(q25.3) 46,XY,der(9)t(9;10)(p24;q22)	36 34
Chromosome 12	12p13.1pter 12q24.3qter 2q37.2qter	5q34qter 12q24.13qter	46,XY,der(11)t(11;12)(p15.3;q12),der(12) t(5;12;11;12)(q34;q12;p15.3;p13.1) 46,XX,del(12)(q24.3) 46,XY,der(2),t(2;12)(q37.2;q24.13)pat	26 37 17
Chromosome 13	13p13q12 13q22qter 13q31.1qter 13q34qter	18p11.2qter 8q11qter	46,XX,-13,+der(18)t(13;18)(q12;p11.2) 46[a],del(13)(q22) 46,XY,del(13)(q31.1) 46,XY,der(13)t(8;13)(q11;q34)mat	38 14 39 33
Chromosome 14	14q32.11qter 14q32.3qter 2q37.1qter	 14q32.3qter	46,XY,del(14)(q32.11) mos46,XX[36%]/46,XX,del(14)(q32.3)[64%] 46,XX,der(2)t(2;14)(q37.1;q32.3)mat	40 41 16
Chromosome 15			46, XX, t(6;15)(p11.2;p12)	30
Chromosome 17	17q21.3q23 17q21.3q24.2 17q22q23.3		46,XX,del(17)(q21.3q23) 46,XY,del(17)(q21.3q24.2) 46,XY,del(17)(q22q23.3)	42 43 44
Chromosome 18	 13p13q12	4p15.32pter 18p11.21qter 18p11.2qter	47,XX,+der(18)t(4;18)(p15.32;p11.21) 46,XX,-13,+der(18)t(13;18)(q12;p11.2)	22 38
Chromosome 22	22q11.2		46,XX,del(22)(22q11.2)	45
Chromosome Y	Yp11.3pter Yq12qter		46,X,rY(p11.3q12)	46

Trisomies and triploidy are not included in the table.
[a] *Sex unknown.*
[b] *Breakpoints not specified.*

Trisomies

OA and/or TOF are found in around 0.5 - 1.0% of children with trisomy 21 (Down syndrome) and up to 25% of children with trisomy 18 (Edwards syndrome).[10,14,47,48] Two reports describe cases of OA/TOF with an almost-complete duplication of chromosome 18 as a result of a translocation involving chromosomes 18 and 4 in one case, and 18 and 13 in the other (see Table 2).[22,38]

Furthermore, OA/TOF was also found in patients with trisomy 13 (Patau syndrome) and (mosaic) trisomy X.[1,8,10,14,49]

The causative mechanism for the anomalies in these trisomies is unknown. A recent paper points at lowered cholesterol synthesis as a possible cause of the spectrum of anomalies seen in trisomy 18.[50] Interestingly, cholesterol is needed for proper functioning of Sonic hedgehog, a gene implicated in the development of the foregut, as described below. [51,52]

Structural chromosomal abnormalities

Partial trisomy 3p

Partial trisomy 3p has been reported in three cases of OA/TOF, with 3p25pter as the common duplicated region. All cases had multiple congenital anomalies with dysmorphisms and cerebral anomalies in all and cardiac and genital anomalies and single umbilical artery in two each.[18-20]

Partial trisomy of the short arm of chromosome 3 is the topic of more than 35 other reports (including 2 siblings of a child with OA), but none of these describe OA/TOF.[1,18,53-60] Yet, anomalies possibly related to OA/TOF are described in two cases, namely tracheal stenosis in a child with a duplication of 3p23pter and a deletion of 5p15.3pter[61] and a hypoplastic larynx and lungs (in addition to multiple other anomalies) in a boy with a trisomy 3p26pter and a trisomy 14pterq24.[62]

Partial deletion 5p

Three reports describe the association of OA/TOF with a deletion of 5p.

Schroeder et al. in 1986 published a case report about a patient with OA, tetralogy of Fallot, and holoprosencephaly with premaxillary agenesis, who had a partial monosomy 5p (p15pter) and a partial trisomy 5q (q32qter).[25] Partial monosomy 5p in combination with OA/TOF was described in two other patients, one of whom also had a partial trisomy 3p23pter, dysmorphic features and complex cerebral malformations, including holoprosencephaly[19] The other had cri-du-chat syndrome as well as Goldenhar syndrome. The cri-du-chat syndrome is a well-known syndrome resulting from a deletion of 5p15.2, but OA/TOF is not usually described as a feature of this syndrome. To the best of our knowledge, this is the only case of cri-du-chat syndrome and OA/TOF published in the literature. Chromosomal analysis showed a terminal deletion of chromosome 5p with

the breakpoint in 5p14.[24] Other reports of terminal deletions of chromosome 5p do not refer to OA/TOF.[14,63,64] However, as mentioned above, Schwanitz and Zerres described a del 5p15.3pter and a dup 3p23pter in association with tracheal stenosis, which can be considered a related anomaly.[61] Kanamori et al. published a case of laryngeal atresia in association with a deletion of 5p13pter and a duplication 16q22qter.[65]

Distal 13q deletion

Distal 13q deletion syndrome has been well documented with more than 140 cases published.[14,39] Phenotypes vary widely and many patients have features of the VACTERL association.[66] Three cases of distal 13q deletion and OA/TOF have been described, their smallest common deletion being 13q34qter. Features of the VACTERL association were found in two of them.[33,39] The third patient was reported by Schinzel from personal observation; further details on additional anomalies are lacking.[14]

Interstitial deletions of 17q

Interstitial deletions of the long arm of chromosome 17 have been described in three cases of OA/TOF. Marsh et al. found a deletion 17q22q23.3 in a child with OA and both a proximal and a distal TOF, as well as limb defects, cardiac anomalies, anal stenosis and dysmorphic features.[44] Dallapiccola et al. in 1993 described del(17)(q21.3q24.2) with OA, TOF, atrial septal defect and ventricular septal defect.[43] TOF, combined with brachycephaly, club feet, dysmorphic features, hand deformities and a delay in growth and development were documented in a child with a del(17)(q21.3q23).[42]

Other papers describing interstitial deletions of chromosome 17q do not mention OA or TOF.[14,67-69] However, the child described by Levin et al. displayed some features of the VACTERL association (vertebral, cardiac and limb defects),[68] and two other patients showed various congenital anomalies, but could not be qualified as having VACTERL association, as only one VACTERL-anomaly (proximally placed thumbs) was present.[67,69]

In contrast to the other chromosomal regions described, this region contains three genes that have been implicated in the development of OA/TOF: *NOG, RARα* and *Tbx4.*

NOG is located on chromosome 17q22 in humans.[70] Animal studies demonstrated its involvement in foregut morphogenesis. Null mutant mice develop OA/TOF in around 70% of cases. However, mutations in *NOG* have not been associated with OA/TOF in humans.[71]

Compound mice deficient for *RARα* (Retinoid acid receptor alpha) and other subtypes of retinoic acid receptors display abnormalities in the separation of trachea and oesophagus, in addition to many other abnormalities.[72] *RARα* is located on 17q21.1 in humans,[73] adjacent to the deletion described in the three patients with OA/TOF, and it could well be that the aberration influenced the expression of RARα.

The *T-BOX (TBX)* genes are a family of transcription factors that play important roles in the development of different species.[74,75] In mice, transient misexpression of *Tbx4* in the prospective oesophageal-respiratory region results in failure of formation of the tracheo-

oesophageal septum.[76] In humans, *TBX4* is located on chromosome 17q21-22.[77] It is conceivable that the deletions on human chromosome 17 described above influence the expression of *TBX4*, e.g. by interfering with a promoter region.

Specific genes implicated in the aetiology of syndromic OA/TOF

Table 3 presents an overview of genes implicated in syndromic OA/TOF. In some cases, such as *SOX2* and *CHD7*, microdeletions found in patients led to the identification of these genes in the aetiology of syndromic OA/TOF, whereas in others, high-resolution techniques, such as array-CGH detected mutations. These genes have recently been reviewed in detail by Brunner et al. and Shaw-Smith.[11,12]

Feingold syndrome

Celli et al. and Van Bokhoven et al. studied Feingold syndrome, which is characterised by microcephaly, digital anomalies, intestinal atresias and short palpebral fissures. OA occurs in around one-third of patients.[78] They found that deletions and mutations of the *MYCN* gene on 2p24.1 are associated with this type of syndromic OA.[78,79]

Anopthalmia-Esophageal-Genital syndrome

Anopthalmia-Esophageal-Genital (AEG) syndrome is an infrequently occurring combination of malformations that has been linked to the *SOX2*-gene on 3q26.3-q27. Deletions and mutations in this gene have been described both in patients displaying OA/TOF and those showing signs of AEG syndrome without OA/TOF, although the majority of cases with this syndrome has OA/TOF as a feature.[80]

CHARGE syndrome

Deletions and mutations in *CHD7* (Chromodomain Helicase DNA-binding protein 7) on 8q12.1 were shown to be a cause of CHARGE syndrome (Coloboma, Heart defects, Atresia choanae, Retardation of growth and development, Genital anomalies in males, Ear anomalies and deafness).[81] Although OA/TOF is not a key feature of CHARGE syndrome, it occurs in around 10% of patients. OA/TOF was found in around 17% of cases of CHARGE syndrome with a mutation in *CHD7*.[29,81-83] The CHARGE phenotype can also be caused by mutations in the *Semaphorin-3E* gene on chromosome 7q21.1, but anomalies of this gene have not been described in patients with OA/TOF.[84]

X-linked Opitz syndrome

Mutations in the *MID1* gene on Xp22 are a cause of X-linked Opitz syndrome, in which oesophagolaryngotracheal defects, including TOF, have been described.[85-88]

22q11.2 deletion syndrome

OA/TOF is sometimes described in patients with 22q11.2 deletion syndrome. The major candidate gene for the anomalies found in patients with this syndrome is *TBX1*.[11,45,89-91]

Table 3 Specific genes implicated in the aetiology of syndromic OA/TOF

Locus	Syndrome	References
MYCN	Feingold	78, 79
SOX2	Anopthalmia-oEsophageal-Genital	80
CHD7	CHARGE	81, 83
MID1	X-linked Opitz	85-87
22q11.2	22q11.2 deletion syndrome	11, 45
GLI3	Pallister-Hall	11, 92-94
FANCA or **FANCC**	Fanconi anaemia	95, 96
PTEN	-	97

Pallister-Hall syndrome

Mutations in *GLI3* were found to be the cause of Pallister-Hall syndrome. In this syndrome, OA/TOF is described only very rarely, but related anomalies such as bifid epiglottis and laryngeal cleft are more common.[11,92,93,98]

Fanconi anaemia

OA/TOF and other features of the VACTERL association, sometimes in combination with hydrocephalus (VACTERL-H), can be part of the spectrum of anomalies seen in Fanconi anaemia. Mutations in the *FANCC* (9q22.3) and *FANCA* (16q24.3) genes causing Fanconi anaemia have been described in patients with Fanconi anaemia and features of VACTERL-H, including OA/TOF.[12,95,96] Based on the low frequency of occurrence of Fanconi anaemia, mutations in these genes would not account for a large proportion of the aetiology of OA/TOF. However, they might explain a small group of cases.[12]

PTEN

A mutation in the *PTEN* gene on 10q23.31 has been described in a patient with VACTERL-H association, including TOF, macrocephaly, ventriculomegaly and malformations of both hands.[97]

Anomalies described in fewer than three cases

Although the more frequently described loci seem to be the most interesting ones from an aetiological point of view, it is important to document rarer anomalies as well. Phenotypical descriptions combined with data from hospital- or population-based databases and, if necessary, repeated cytogenetic testing with more detailed techniques, could identify chromosomal regions, and later possibly specific genes, responsible for OA/TOF in specific subgroups of patients. Other papers describing the same chromosomal anomalies may help determine if a specific aberration causes a distinct

pattern of anomalies. Genetic mechanisms such as variable expression may complicate the analysis of these chromosomal defects.

As can be appreciated from table 2, the regions described in two cases are: distal 2q,[15,16] distal 4q,[20,23] distal 5q,[25,263] proximal 6q,[27,28] distal 8q[31,33] and distal 14q.[40,41] Triploidy has also been described in association with OA/TOF in two cases.[1,99]

Although reported in only one case, terminal deletion of chromosome 7q is worth a brief comment. A girl with a deletion of 7q34qter and a duplication of 8q24.1qter showed OA with a distal TOF in addition to growth retardation, microcephaly, eye, limb, skeletal and renal anomalies.[31] The deleted region contains *Sonic hedgehog*, a gene involved in foregut development.[100] However, quite a few reports describe cases with distal 7q deletion in the absence of oesophageal anomalies, which for now makes it hard to assign a direct causal link between deletions of *Sonic hedgehog* and the development of OA/TOF in humans.[14,101,102]

Loss and gain of material on the same locus

There are four chromosomal loci on which both gain and loss of material have been described, namely proximal 8q (trisomy 8q11qter and deletions of the *CHD7* gene),[33,81-83] distal 10q,[34,36] distal 12q[17,37] and distal 14q.[16,40,41] These findings may seem contradictory. A possible explanation is that the genes in these regions are dosage-sensitive. A disturbance, be it under- or overexpression of the gene, may result in faulty development of one or more organ systems, thereby causing similar congenital anomalies.

Balanced translocations

Apparently balanced translocations in individuals with OA/TOF may lead to the identification of a causative gene, either through the disruption of a gene by the breakpoint, or, as recently illustrated, by the occurrence of a breakpoint-associated submicroscopic deletion.[103,104] A recent paper described a translocation between chromosomes 6 and 15, in which the breakpoint on chromosome 6 was within the *BPAG1* gene, thereby selectively disturbing two specific isoforms of the protein, which may explain the phenotypical features, including OA/TOF.[30]

DISCUSSION

OA/TOF may occur as an isolated anomaly, it may be part of a complex of congenital defects or it may develop within the context of a known syndrome or association. Genetic factors are most likely to play a role in the latter two groups. Detailed description and comparison of cases of OA/TOF, combined with (cyto)genetic testing may lead to the discovery of specific loci or genes with a possible causative role. Techniques such as Fluorescent *In Situ* Hybridisation (FISH), DNA-based Comparative Genomic Hybridisation and whole genome bac or oligo arrays have made it possible to find small anomalies that

may have gone undetected in earlier days. Now that various chromosomal regions have been implicated in the development of OA/TOF, re-examination of the karyotypes of children diagnosed earlier with OA/TOF and a normal karyotype, with a specific focus on these regions, might be indicated.

We have not been able to find specific regions linked to TOF, nor to OA with or without a TOF. As the term TOF (or TEF in the American literature) is often used both for fistulas and atresias, it is possible that authors of the case reports we reviewed reported TOF in their cases, when in fact the child had an atresia, making it hard to get a good overview of the exact anomalies present.

As the search for genes relevant for specific anomalies continues to link new genes to these anomalies, this overview of chromosomal anomalies in children with OA/TOF may help in the identification of chromosomal regions involved in the aetiology of this major congenital anomaly.

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Part II

Phenotypic Aspects

Non-VACTERL-type anomalies in Patients with Oesophageal Atresia / Tracheo-oesophageal Fistula and full or partial VACTERL association.

Chapter 4

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ABSTRACT

Background
The VACTERL association is the non-random co-occurrence of *V*ertebral anomalies, *A*nal atresia, *C*ardiovascular malformations, *T*racheo-oesophageal fistula (TOF) and/or o*E*sophageal atresia (OA), *R*enal anomalies and/or *L*imb-anomalies. The full phenotype of patients with OA/TOF and VACTERL association is not well described in the literature.

Methods
Data on patients with OA/TOF seen in two paediatric 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 of the defects of the VACTERL spectrum in addition to OA/TOF was the criterion for inclusion in this study. A detailed description was made of all defects.

Results
Of 463 patients with OA and/or TOF, 109 (23.5%) fulfilled the inclusion criterion. Seventeen cases had recognized causes for their anomalies and were excluded, leaving 92 cases (19.9%) for analysis. Other than the oesophagus and the trachea, the vertebrae/ribs and the cardiovascular system were most commonly affected (67.4 and 66.3%, respectively). Interestingly, 70.7% of cases had additional non-VACTERL-type defects, with high occurrences for e.g. single umbilical artery (19.6%), genital defects (23.9%) and respiratory tract anomalies (14.1%).

Conclusions
Many patients display non-VACTERL-type congenital anomalies in addition to defects of the VACTERL spectrum.

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 Vertebral defects, Anal atresia, Tracheo-oEsophageal fistula (TOF) and/or oesophageal atresia (OA), Renal anomalies and Radial dysplasia. Later, cardiac (C) and limb (L) defects were added, so that the acronym extended to VACTERL.[2] The inclusion of cardiac defects is still debated, however, and so is the number of defects (two or three) needed for the diagnosis.[3-8] Reported incidences fall in the range of 0.3 to 2.1 per 10,000 live births, depending on the clinical definition used and the population studied.[3,4,7]

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.[4,7,9]

The aetiology 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 a causative role in the aetiology of this association.[10-13]
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.[14]
Environmental exposures during pregnancy, such as exogenous sex hormones during the first trimester of pregnancy, have been suggested as risk factors,[2] but no single environmental risk factor has consistently been identified.[8,15-17]
When Adriamycin, an antibiotic used in chemotherapy, is given to pregnant rats, it produces many anomalies of the VACTERL-type in the offspring.[18] So far, however, Adriamycin or structurally related substances have not been found associated with VACTERL-type anomalies in humans.[19-21]

Reported proportions of patients with full or partial VACTERL association featuring OA/TOF range from 24 to 67%.[4,7,9] Only four papers, however, describe this specific group of patients.[22-25] Previous studies describe small sample size,[22,23] do not describe non-VACTERL-type defects,[25] or use a wide definition of VACTERL association.[24]

In this study, we evaluated a large population of patients with OA/TOF for the occurrence and characteristics of defects of the VACTERL spectrum and of non-VACTERL-type structural malformations in particular. Our rationale was to provide clinicians and researchers with a good overview of these patients, who may share a common, but so far unknown, aetiology.[26]

METHODS

We evaluated data stored in the databases of the Erasmus MC - Sophia Children's Hospital in Rotterdam and the Paediatric Surgical Center of Amsterdam (Emma Children's Hospital AMC and VU University medical center), both in the Netherlands. The paediatric surgical departments of these university hospitals are among the six tertiary referral centers for paediatric surgery in the Netherlands. The Sophia Children's Hospital is the paediatric referral center for the Southwestern part of the Netherlands and has the only specialized paediatric surgical intensive care unit in the Netherlands. The Paediatric Surgical Center of Amsterdam is the referral center for paediatric 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 patients with OA/TOF from the referral areas are admitted to either of the two hospitals. Both centers keep comprehensive databases on cases with OA/TOF, including data on associated anomalies, pregnancy history, and postnatal clinical course.

We used the documented presence of at least two other VACTERL-type defects (*i.e.*, in addition to OA/TOF), namely, vertebral anomalies (including rib anomalies), anal atresia, cardiovascular malformations, renal anomalies or limb anomalies as a criterion for the diagnosis of VACTERL association.

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

Data from all patients who fulfilled the inclusion criteria, as defined above, were extracted from the databases and medical records were checked to confirm the diagnosis and the presence of associated anomalies. Data were analyzed using SPSS 12.0.1.

RESULTS

A total of 463 patients with OA and/or TOF had been admitted to either of the two centers from January 1988 to August 2006. Of these, 109 (23.5%) patients had two or more other VACTERL-type defects. Seventeen patients had recognized causes, such as syndromes or chromosomal disorders, and were excluded (Table 1). This left 92 (19.9%) cases for analysis, which included 58 (63.0%) males and 34 (37.0%) females. Using the Gross classification, we identified four (4.3%) cases with type A (isolated OA), one case (1.1%) with type B (OA with proximal TOF), 83 (90.2%) with type C (OA with distal TOF), two cases (2.2%) with type D (OA with proximal and distal TOF) and one (1.1%) case with TOF without OA (type E).[27] One patient with type C had two distal fistulas. The type was not recorded in one patient.

Table 1 Chromosomal anomalies & syndromes found in 109 cases with OA/TOF fulfilling the criteria for VACTERL association

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-six cases (39.1%) were born preterm (< 37 weeks) and six (6.5%) were post term (42 weeks or more). Birth weights ranged from 775 to 3825 g (mean 2422 g). Birth weight for gestational age showed that 25 cases (27.2%) 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 26 patients (28.3%) died, of whom 13 (50.0%) at neonatal age (median age of death 4 days, range 0 - 21 days) and 12 (46.2%) at infant age (median age of death 177 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 26 non-survivors had all six anomalies of the VACTERL spectrum (full VACTERL association), three had five anomalies, five had four anomalies and 17 had three. Twenty-three non-survivors had cardiac defects, of whom seven had more than one. Five patients had severe anomalies of the respiratory tract that may have contributed to their deaths – e.g. tracheal agenesis in one case - 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.

Table 2 gives an overview of the VACTERL-type defects other than OA/TOF found in the 92 analyzed cases. The vertebrae/ribs and the cardiovascular system were most commonly affected (67.4 and 66.3%, respectively). Of the 92 cases, 61 (66.3%) had three defects, 22 (23.9%) had four defects, seven (7.6%) had five defects and two (2.2%) patients were diagnosed with all six VACTERL components.

Table 2 Defects of the VACTERL association found in 92 cases with OA/TOF

Defect	No. of cases (%)
Vertebral	**62 (67.4)**
Vertebral anomalies	50 (54.3)
Rib anomalies	37 (40.2)
Anal	**38 (41.3)**
Anal atresia/stenosis/web	31 (33.7)
Abnormal placement of anus	7 (7.6)
Cardiovascular	**61 (66.3)**
Atrial septum defect	33 (35.9)
Ventricular septum defect	25 (27.2)
Atrio-ventricular septal defect	4 (4.3)
Fallot tetralogy	9 (9.8)
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 (34.8)**
Hydronephrosis	7 (7.6)
Horseshoe kidneys	7 (7.6)
Renal agenesis	8 (8.7)
Renal cysts	6 (6.5)
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	**31 (33.7**)
Radial anomalies	16 (17.4)
Thumb anomalies	18 (19.6)
Polydactyly	6 (6.5)
Syndactyly	3 (3.3)
Clinodactyly	2 (2.2)
Brachomesophalanx dig.5	1 (1.1)
Hypoplastic or absent carpals	1 (1.1)

Defects described are not mutually exclusive

Interestingly, while only 27 cases (29.3%) just had VACTERL-type defects, as many as 65 cases (70.7%) had VACTERL-type defects in association with other structural defects (Table 3). A high occurrence of single umbilical artery (19.6%) and genital defects (23.9%) was observed. Respiratory tract anomalies were seen in 13 (14.1%) patients. Duodenal atresia and cleft lip/jaw/palate were seen in eight (8.7%) and four (4.3%) patients, respectively.

DISCUSSION

This Paediatric Surgical Center-based study describes a group of 92 patients with OA/TOF and at least two other defects included in the VACTERL spectrum. The defects are well documented, thanks to the routine screening of children with OA/TOF for VACTERL-type defects with more detailed, mainly non-invasive, diagnostic techniques (e.g., renal and cardiac ultrasound) since the late 1980s.
The most remarkable finding in our population was that as much as 70.7% of all patients showed defects other than those included in the VACTERL association. The corresponding figure reported by Chittmittrapap et al. is only 52%.[22]

In a study of VACTERL association in general, including all cases with any three or more of the six defects, non-VACTERL-type anomalies were described in 20%.[7] The high numbers reported in our population may reflect the high percentage of patients with OA/TOF that have associated anomalies in general (around 50%).[22,28] In addition, our population is very well documented and many cases have been assessed by a clinical geneticist/dysmorphologist, increasing the likelihood of more minor anomalies being noticed and documented. However, most non-VACTERL-type anomalies found are not such minor anomalies that we would expect them to be missed on routine clinical examination (Table 3).

Comparing our results to those of others is not an easy task. Several of the defects associated with OA/TOF are also associated without the presence of OA/TOF.[3,6,7] However, we focused on defects associated with OA/TOF and only describe those patients with full or partial VACTERL association who apart from OA/TOF have at least two other VACTERL-type defects. There are only a few papers in the literature reporting data from populations similar to ours.[22-25]

In our population, 19.6% of cases had a single umbilical artery (SUA). The corresponding figure reported by Chittmittrapap et al. was 8%, but Temtamy and Miller, in a smaller group, described an incidence of 70%.[23] In a study based on data for 292 cases of OA/TOF in the United States, Torfs et al. reported a strong association between SUA and OA/TOF in general, with SUA found in 18.2% of cases with OA/TOF.[25] This population, however, included 36 cases with aneuploidies and syndromes. Nevertheless, none of the 28 trisomy cases had a SUA[25] and exclusion of these cases would therefore bring up this proportion to 20%, which is in line with our finding.

Table 3 Associated non-VACTERL-type structural anomalies found in 92 cases with OA/TOF

Anomaly	Number of cases (%)
Single umbilical artery	**18 (19.6)**
Duodenal atresia/web	**8 (8.7)**
Cleft lip/jaw/palate	**4 (4.3)**
Genital anomalies	**22 (23.9)**
Testicular anomalies, including cryptorchidism	10 (10.9)
Penile anomalies, including hypospadias	9 (9.8)
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 (15.2)**
Vesico-urinary reflux	11 (12.0)
Patent urachus	1 (1.1)
Bladder anomalies	3 (3.3)
Urethral anomalies, including valves	3 (3.3)
Respiratory system anomalies	**13 (14.1)**
Laryngeal/tracheal/bronchial anomalies	7 (7.6)
Lung hypoplasia/agenesis	2 (2.2)
Lung lobulation defects	1 (1.1)
Cricoid stenosis	1 (1.1)
Choanal atresia	1 (1.1)
Uvular anomalies	2 (2.2)

Intestinal anomalies	**9**	**(9.8)**
Intestinal malrotation	4	(4.3)
Small bowel atresia/hypoplasia	1	(1.1)
Meckel's diverticulum	4	(4.3)
Lower limb anomalies	**17**	**(18.5)**
Hypotrophy lower limbs	1	(1.1)
Dysplastic hip(s)	2	(2.2)
Club foot	4	(4.3)
Rocker-bottom feet	1	(1.1)
Anomalies of toes	12	(13.0)
Hallux valgus	1	(1.1)
Skeletal (other)	**12**	**(13.0)**
Upper limbs, non-VACTERL defects	10	(10.9)
Abnormally shaped thorax	2	(2.2)
Skull anomalies	1	(1.1)
Vascular anomalies (other)	**10**	**(10.9)**
Abnormal arterial supply right lung	1	(1.1)
Anomalies of vena cava	5	(5.4)
Right descending aorta	3	(3.3)
Anomalous venous return	2	(2.2)
Nervous system anomalies	**10**	**(10.9)**
Anomalies of spinal cord, including spina bifida	6	(6.5)
Structural brain anomalies	5	(5.4)
Other anomalies	**6**	**(6.5)**
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

We found duodenal atresia in 8.7% of our population and cleft lip, jaw and/or palate in 4.3%. The only other study with data comparable to ours that reports on the occurrence of these defects describes duodenal atresia in a similar proportion of cases (8.0%), but no cases of clefts.[22] In studies of associated anomalies in children with OA/TOF, cleft lip and palate were reported in 2.6% and 2.7% of patients.[29,30] 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.[24]

Anomalies of the respiratory system were present in almost 15% of cases. The finding of tracheal and/or lung defects in this group of patients is not entirely surprising, as both the oesophagus and the respiratory system develop from the foregut. The presence of OA/TOF may be a signal of defective foregut development in general, which could also lead to respiratory tract anomalies. A number of mouse knockout models that show OA/TOF also display anomalies of the respiratory system, e.g. knockout mice for *sonic hedgehog*, *gli2/gli3* or *Ttf-1.*[31-33] In a group of 25 cases with OA/TOF and VACTERL association, Chittmittrapap et al. described absence of the right upper lobe of the lung in one patient (4%).[22] Temtamy reported unilateral lung agenesis in a similar, but smaller, group (n = 10) in an unspecified number of cases.[23]

We found a 19.9% prevalence of combinations of at least three VACTERL-type defects (including OA/TOF), which falls in the range of prevalences reported by other groups (10 - 32%).[22,23,25,34] We found 63.0% of all these cases to be boys, which compares with the proportion reported by Chittmittrapap et al.[22]

Only a small proportion of patients had the whole spectrum of six anomalies, thereby representing the "full" VACTERL association. Although some other studies on the VACTERL association include patients with no more than two of the anomalies of the VACTERL spectrum,[3,9,16] most report only those patients with three or more defects.[4,6,8,22,23] We have adopted the latter, more stringent definition.
Therefore, most patients in our study have "partial" VACTERL association. Our data do not permit to draw conclusions about the number of defects needed for the diagnosis.

Also, some studies exclude patients who fulfill the criteria for VACTERL association, but who have been diagnosed with a specific chromosomal anomaly or syndrome.[7,9] The same decision was made in the present study. Others, however, have decided to include these cases, which makes comparison more difficult.[24]

Karyotyping is now becoming standard practice for children with congenital anomalies in many centers. 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 aiming to exclude children with chromosomal anomalies may have included children with such anomalies that less sensitive techniques could not detect. In the future, when more information is available on chromosomal anomalies

possibly related to (full or partial) VACTERL association, it might be indicated to re-examine the children diagnosed earlier with a normal karyotype, both clinically by a clinical geneticist, as well as by using more detailed techniques, such as array-based Comparative Genomic Hybridization.[35] This approach has been successfully implemented in other major congenital anomalies, such as congenital diaphragmatic hernia.[36]

In summary, this study describes phenotypes of patients with OA/TOF and full or partial VACTERL association treated in two large paediatric surgical centers. Their clinical data are well-documented in comprehensive databases. 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. Importantly, it shows that almost 71% of patients display non-VACTERL-type congenital anomalies in addition to defects of the VACTERL spectrum.

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Agenesis of the trachea: Phenotypic expression of a rare cause of fatal neonatal respiratory insufficiency in six patients

Chapter 5

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ABSTRACT

Tracheal agenesis is a rare congenital malformation, which is usually fatal in the newborn period. Its incidence is approximately 1 in 50,000 births. Presentation is with respiratory insufficiency and no audible cry. Other anomalies are found in most cases. Six cases of tracheal agenesis were seen in our hospital since 1988. Their medical records were reviewed. Three of our cases classify as Floyd's type III, two as Floyd's type II and one as Floyd's type I. Associated anomalies were found in five cases. The classification of tracheal agenesis, associated anomalies and potential therapeutic options are discussed.

INTRODUCTION

Tracheal agenesis is a rare congenital malformation of the respiratory tract. The diagnosis may be suspected in newborns with respiratory insufficiency, absence of audible crying and difficult endotracheal intubation and ventilation. Occasionally roentgenographic examination demonstrates absence of a tracheal air column.[1]

Tracheal agenesis is usually fatal in the neonatal period, with no established methods for surgical repair at this point in time. The longest surviving child described in the literature died at the age of 6 years and 10 months.[2] Approximately one in 50,000 live births is reported to suffer from agenesis of the larynx, trachea or bronchi.[3,4] Tracheal agenesis is often associated with other congenital anomalies: previous studies found associated defects in 50-94% of patients.[1,5] Boys are affected approximately twice as often as girls.[5,6] Since the first description in 1900,[7] approximately 150 cases of tracheal agenesis have been reported worldwide as reviewed by Hirakawa et al.[8]

CASE REPORTS

Six patients with tracheal agenesis were seen in our tertiary referral hospital (Erasmus MC- Sophia Children's Hospital, Rotterdam, the Netherlands) since 1988. All but one (case no. 6) underwent diagnostic laryngobronchoscopy and/or oesophagoscopy and autopsy was performed in all cases. General patient characteristics are summarized in Table 1 and an overview of the associated anomalies is presented in Table 2.

All cases presented with severe respiratory distress after birth. Intubation was difficult or impossible. Patients 1, 3 and 4 were ventilated through the oesophagus with reasonable results for a limited amount of time, but long-term ventilation was impossible. Tracheotomy was tried in cases 2 and 5. In the former, a floppy air-filled space was found, consisting of "trachea" and oesophagus. More distally, this structure divided into a "trachea" on the left, leading to the lungs, and an oesophagus on the right. By ventilation through two tubes, one placed in the "trachea" and one in the oesophagus (Figure 1), oxygen saturations of over 90% were achieved, but they progressively decreased there-after. In the latter, no trachea could be identified at all. Resuscitation of case 6 after birth failed and she died shortly postpartum.

The results of diagnostic (oesophago)laryngobronchoscopies and autopsies are described in the following section.

Table 1 Patient characteristics

Case no.	Gender	GA (w+d)	Birth weight (g)	Type TA	Apgar scores	Survival	Karyotype	Other
1	F	39+2	3,040	Floyd III	3-8	10h	-	Polyhydramnios
2	M	30+1	1,460	Floyd III	6-5-4	Several h	46,XY	Prolonged ROM Maternal fever Anhydramnios Breech presentation
3	F	34+5	2,110	Floyd II with TOF	4-4-5-6	18h	46,XX	Polyhydramnios Caesarean section Breech presentation
4	M	28+2	1,020	Floyd III	5-9-9-8	Several h	46,XY	-
5	M	38+2	2,010	Floyd II, no TOF	1-1	20 min	46,XY	Prenatal diagnosis of multiple anomalies Induction of labour
6	F	30+5	1,180	Floyd I with TOF	1	15 min	46,XX	Polyhydramnios Breech presentation 2^{nd} of twins, other child healthy

F: female, M: male, GA: gestational age, w: weeks, d: days, g: grams, TA: tracheal agenesis, Apgar scores are scores after 1-5-10 and 20 minutes respectively, if applicable, h: hours, min: minutes, TOF: tracheo-oesophageal fistula, ROM: rupture of membranes

Table 2 Associated anomalies

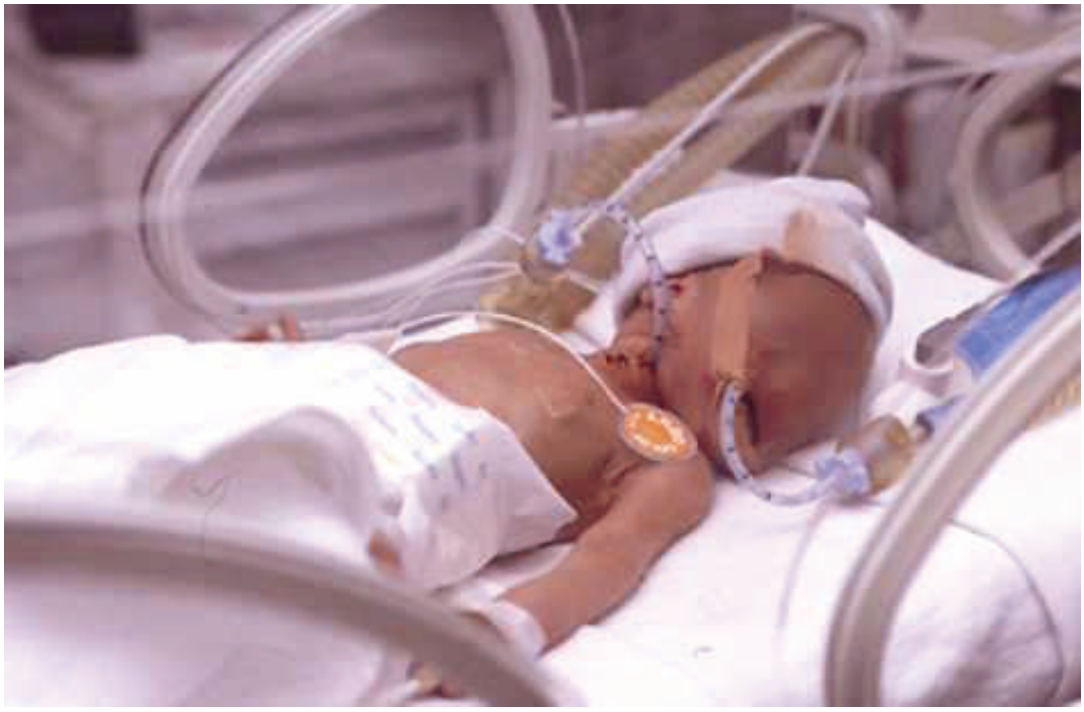
Case no.	Skeletal	Extremities	Laryngeal	Gastrointestinal	Cardiac	Renal	Urogenital	Other
1	-	-	+	-	-	-	-	-
2	+	-	+	+	+	+	+	+
3	-	-	+	+	+	-	-	+
4	-	-	+	+	+	-	-	+
5	-	+	-	-	-	-	+	+
6	-	+	-	-	-	-	-	+

+: present, -: absent

Case no. 1 (This case was previously describesd by Manschot et al.[9])
Emergency rigid laryngobronchoscopy showed a normal hypopharynx, epiglottis and arytenoids. Immediately distally from the vocal cords, a complete tracheal agenesis with a blind ending larynx was seen. Oesophagoscopy revealed a fistula at approximately 45 mm from the pharyngo-oesophageal junction.

Autopsy confirmed tracheal agenesis. There were two broncho-oesophageal fistulas, located on the left and the right side, seven centimeters distally from the epiglottis and a 10-mm dorsal laryngeal cleft. No other congenital anomalies were found.

Figure 1 (*for colour figure see page 221*)



Case no.2, ventilated through two tubes, one tube was inserted orally and the other through the tracheostomy.

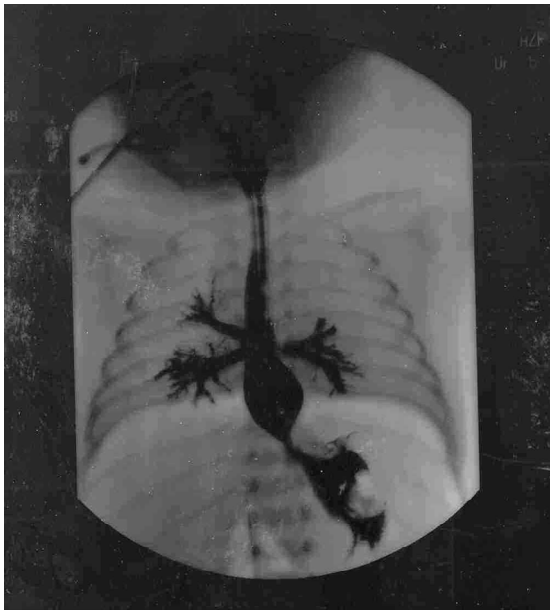
Case no. 2
Emergency laryngobronchoscopy showed an abnormal larynx with a small, flat and floppy epiglottis. More distally, a large pouch with collapsing walls was seen. There were no tracheal rings and no carina could be found. Oesophagoscopy revealed a fistula arising from the ventral side of the oesophagus at three to four centimeters distally from the pharyngo-oesophageal junction.

Postmortem X-rays showed low-thoracic and high-lumbar hemivertebrae and rib anomalies. Autopsy confirmed tracheal agenesis with two bronchi arising separately from the oesophagus. It further revealed a proximal duodenal atresia, agenesis of the gallbladder, Meckel's diverticulum, a secundum-type atrial septal defect and horseshoe kidneys with mild hydro-ureters bilaterally. No structural anomalies of the brain were found.

Case no. 3
Diagnostic laryngobronchoscopy showed a small epiglottis with no lumen from the subglottic region downwards. At oesophagoscopy a fistula was seen, through which the lungs were assumed to be ventilated. Tracheal cartilage rings were not present.

Postmortem examination confirmed the tracheal agenesis. A fistula arose from the oesophagus, connecting it to the main bronchi, as could be seen on a postmortem contrast study (Figure 2). Additional findings were duodenal atresia due to an annular pancreas, intestinal malrotation, a ventricular septal defect, a single umbilical artery, an atrophic thymus and an accessory spleen on the left side. Full-body X-rays showed no skeletal anomalies.

Figure 2 Postmortem contrast study of case no.3



Contrast was administered postmortem through the oesophagus. The oesophagus and the stomach are clearly visible, as well as three bronchi.

Case no. 4
Laryngobronchoscopy showed an infantile epiglottis, normal vocal cords, but no subglottic lumen. In addition, there was a dorsal laryngeal cleft. Oesophagoscopy revealed a ventral fistula at nine centimeters from the lips.

Postmortem examination confirmed tracheal agenesis and the laryngeal cleft. Both bronchi arose separately from the oesophagus. In addition, anal atresia with a recto-urethral fistula and a perimembranous ventricular septal defect with an arteria lusoria were found. No external dysmorphic features were noted, except for hypertelorism. Neuropathological examination showed an immature brain with ongoing neural migration and polymicrogyri.

Case no. 5
Laryngobronchoscopy and oesophagoscopy showed a normal larynx, ending blindly at the subglottic level and agenesis of the trachea, but a normal oesophagus. No fistula was seen.

Postmortem examination showed mild asymmetry of the skull, mildly abnormal feet and a prune belly-like appearance of the abdomen. There was an agenesis of the trachea of approximately three centimeters in length from two centimeters below the epiglottis downwards. No structural pulmonary anomalies were found. The heart was relatively large, without any obvious structural defects. The oesophagus and abdominal organs were normal, except for a unilateral undescended testis. Neuropathological examination showed no structural anomalies of the brain.

Case no. 6
No laryngobronchoscopy or oesophagoscopy were performed in this patient. At autopsy, tracheal agenesis with a distal tracheo-oesophageal fistula was seen. No other oesophageal anomalies were noted. Both main bronchi could be entered through the fistula. In addition, the right thumb was missing and the left hand was malformed with four digits. The left ear was dysmorphic. No structural cardiac anomalies were diagnosed. The brain was structurally normal.

DISCUSSION

To the best of our knowledge, this is the largest case series of tracheal agenesis from a single hospital described so far. We present the variation in phenotypic expression and the spectrum of associated anomalies of tracheal agenesis as a neonatal emergency.

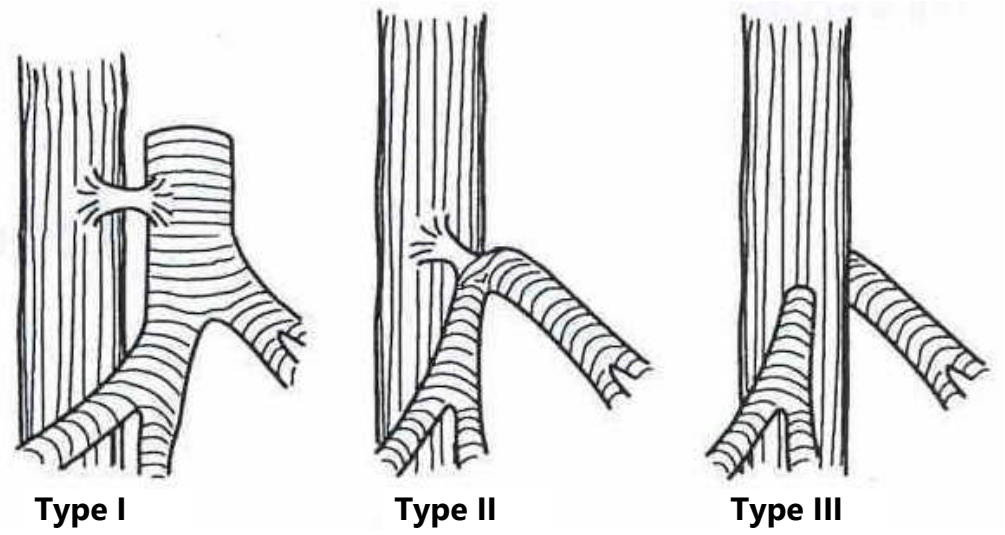
Classification
Tracheal agenesis is usually classified according to Floyd into types I - III (Figure 3).[10] Type I is characterized by agenesis of the proximal trachea, with a short caudal part and a

tracheo-oesophageal fistula. Type II consists of agenesis of the entire trachea with main bronchi joining in the midline. A fistula between oesophagus and carina may or may not be present. In type III, two main stem bronchi arise separately from the oesophagus. An alternative classifying system, using seven different categories (A - G), was later developed by Faro et al.[11,12] Although it is more extensive, the latter system is used less commonly than the former, as subtypes are not as accurately defined as in Floyd's classifying system. The commonest type of tracheal agenesis is Floyd's type II, accounting for approximately 49 - 59% of cases, with type III accounting for approximately 30% and type I for only 10 - 15% of cases.[6,9] Three of the cases (cases 1, 2 and 4) described in this report classify as Floyd's type III, one (case 6) classifies as Floyd's type I and one (case 3) as Floyd's type II with a tracheo-oesophageal fistula. Case 5 classifies either as Floyd's type II without a fistula, or as Faro type G. Absence of a tracheo-oesophageal fistula is a rare finding. Only one of 32 cases of tracheal agenesis reviewed by Bray and Lamb[13] lacked a tracheo-oesophageal fistula.[13] More recently Van Veenendaal et al. reviewed the literature on both agenesis (n = 82) and atresia (n = 7) of the trachea. A tracheo-oesophageal fistula was present in most (94%) of the cases.[6]

Aetiology

At present the aetiology of tracheal agenesis is unknown. Tracheal agenesis, oesophageal atresia, tracheo-oesophageal fistula and posterior laryngotracheo-oesophageal cleft can all be seen as part of a spectrum of anomalies of the foregut. In recent years, major progress has been made in understanding the genetic mechanisms involved in foregut development. Candidate gene approaches using knockout mice have shown that mutations in genes like *Sonic Hedgehog*, *Gli2* and *3* and *Thyroid transcription factor-1* can lead to defects of the foregut derivatives such as trachea and oesophagus.[14-16] However, a specific chromosomal basis for tracheal agenesis in humans has not yet been identified.

Figure 3 Types of tracheal agenesis according to Floyd



(Reprinted with permission from Manschot et al, 1994.[9])

Embryology

The respiratory system develops in five distinct stages, incorporating two processes: lung growth and lung maturation.[17,18]

During the first stage, the *embryonic lung development* (3 - 6 weeks), the foregut divides into trachea and oesophagus. However, the exact mechanism of division and the pathogenesis of abnormal tracheo-oesophageal development is disputed, as reviewed by Kluth and Fiegel[19] and Merei and Hutson.[20] Next, in the *pseudoglandular stage* (7 - 16 weeks), all prospective conductive airways are formed, after which the bronchial structures of the lungs are formed and vascularized during the *canalicular stage* (16 - 24 weeks). The mature alveoli develop during the *saccular stage* (25 weeks to birth) and the *postnatal* or *alveolar stage*.[17,18,21]

Animal studies have shown that tracheal occlusion during the early canalicular stage induces acceleration of alveolar-saccular development.[22] Considering this, the normal appearance of the lungs and lobal pattern, in the absence of a tracheo-oesophageal fistula, as found at postmortem-examination of case 5, was to be expected.

Associated anomalies

Up to 94% of cases of tracheal agenesis are reported to have additional congenital anomalies in various organ systems.[5] Most commonly affected are the cardiovascular system (69%), the respiratory tract distal to the trachea (45 - 64%), the gastrointestinal tract (47 - 50%), the genitourinary tract (35 - 49%), the musculoskeletal system (19 - 38%) and the nervous system (7%).[5,6] Five of the six cases reported in our series indeed had associated malformations. An overview of the associated anomalies in our cases can be found in Table 2.

It has been suggested that tracheal agenesis should be considered a component of the VA(C)TER(L) association, which includes Vertebral defects, Anal atresia (Cardiac anomalies), Tracheo-oEsophageal fistula, Renal anomalies and (Limb defects).[23] However, Evans et al. suggested that in spite of the overlap between the associated anomalies seen in tracheal agenesis and VA(C)TER(L) association, differences in nature and frequency of the anomalies involved distinctly discriminate the two as separate clinical entities.[5]

Cases 2 and 3 seem also to be affected by (at least) three features of another association, the TACRD (tracheal agenesis or laryngotracheal atresia, cardiac abnormalities, radial ray defects and duodenal atresia) association, thereby meeting the criteria for diagnosis.[6,24]

Therapeutic Options

Although emergency management, by either bag and mask ventilation or oesophageal intubation, can at times be successful after definitive diagnosis of tracheal agenesis (as in cases 1 - 4), long-term therapy of this condition remains a problem. Cases hitherto described in the literature have, almost without exception, been fatal. Soh et al. described

a child who survived for 6 years and 10 months. Three other survivors beyond the neonatal period have been described, although no information could be found in the literature about long-term follow-up of these cases.[25,26]

In case of doubt about the exact diagnosis and prognosis, extracorporeal membrane oxygenation (ECMO) can also be considered to buy time while evaluating the possibility of tracheal reconstruction in selected cases.

The search for suitable materials for tracheal grafts has so far been unsuccessful. Possible future grafts will have to allow for normal growth and development, be able to clear the airways from secretions and be able to withstand pressure changes during respiration.

We feel that at present, with no long-term solutions for tracheal agenesis at hand, it would be appropriate to consider minimizing clinical interventions once the diagnosis has been made. Of note is the possibility that both new materials (prosthetic materials, tissue engineering, etc.) and new surgical techniques, such as the Ex-Utero Intrapartum Therapy procedure (EXIT)[27,28] might offer new perspectives for short-term securing of the airway and long-term surgical reconstruction of the trachea in the future. However, an EXIT procedure will only be an option in a few selected cases in which the diagnosis has been made prenatally, in which there is a Floyd type I tracheal agenesis with a caudal trachea with permits surgical intervention and in which there are no life-threatening associated anomalies.

ACKNOWLEDGEMENTS

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Part III

Genetic Aspects

Gene-expression analysis of tracheo-oesophageal fistulas

Chapter 6

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ABSTRACT

Background
Oesophageal atresia (OA) and tracheo-oesophageal fistula (TOF) are relatively frequently occurring foregut malformations with a largely unknown aetiology. To gain more insight into the origin of the TOF, we aimed to examine and describe gene expression patterns in TOFs using an unbiased, whole-genome approach.

Methods
Tissue samples of the TOF taken out during surgery. Control tissue from autopsies of children of 17 - 25 weeks gestational age (lung, trachea and oesophagus) was received from the tissue bank of the Erasmus MC. Affymetrix Human Genome U133A Plus 2.0 microarray chips were run with RNA isolated from these tissues. Data analysis was done with OmniViz software for the SAM analyses and with Ingenuity Pathway Analysis. The Locally Adaptive statistical Procedure (LAP) was used to identify chromosomal regions with differential expression between cases and controls.

Results
21 TOFs, 3 oesophageal, 3 tracheal and 4 lung samples were used. There was a clear difference between TOFs and controls and there were at least two subgroups with the TOF group. TOFs were by far the least similar to lung tissue. Important functions that arose from the analysis of significantly differentially expressed genes between TOFs and trachea and TOFs and oesophagus were cellular development, tissue morphology and tissue development. A number of chromosomal regions with differential expression between TOFs and controls were found.

Conclusions
This study describes a whole-genome, unbiased analysis of gene expression patterns in human TOFs. TOFs seem to represent a specific type of tissue. Based on the number of differentially expressed genes, the TOFs were most similar to oesophagus. The heterogeneity of the TOFs is reflected in their gene expression patterns. A definitive answer regarding the aetiology of pathogenesis of OA/TOF can not be provided by this research.

INTRODUCTION

Oesophageal atresia (OA) and tracheo-oesophageal fistula (TOF) are relatively frequently occurring foregut malformations with an incidence of around 1 in 3,500 births.[1-3] On morphological grounds, five types of oesophageal atresia are recognised, of which proximal atresia with a distal TOF is the most common one, occurring in 85% of cases.[4]
The aetiology of OA/TOF is largely unknown and is considered to be multifactorial. Environmental factors have been suggested to play a role, but no external factor has consistently been identified.[3,5-14] 6 - 10% of patients with OA/TOF have chromosomal anomalies, mostly trisomies.[1,15] Recently, four genes, *MYCN*, *CHD7*, *MID1* and *SOX2*, have been reported to be involved in the aetiology of syndromic OA/TOF.[16-23] Hypomorphic *Sox2* mutant mice have been shown to also display OA/TOF in around 60 - 70% of cases, thereby providing a link between humans and mice.[24]

From knockout studies in mice, a number of genes, such as *Sonic hedgehog* (*Shh*), *Gli2*, *Gli3*, *TTF-1* and *Noggin*, have been implicated in the development of OA/TOF.[25-27] So far, a deletion of *SHH* (7q36) has been reported in one patient with OA/TOF.[28] However, this patient also had an anomaly of chromosome 8 and there are a number of reports describing deletions in *SHH* in cases without OA/TOF, making it hard to assign an aetiological role to *SHH* in humans at this point in time. Mutations in the human *GLI3* gene cause Pallister-Hall syndrome, in which TOF is occasionally described.[29-32] No mutations in the other genes implicated in knockout mice have been described in humans with OA/TOF yet.

The exact mechanisms of normal and abnormal development of the foregut are a subject of discussion in the literature. Several theories have been proposed to explain the mechanism of separation of the ventral respiratory and dorsal oesophageal parts of the foregut, but controversy still exists.[33-40]
Histological studies of the TOF and distal oesophagus show a mixed picture. Human samples have been described to have (pseudo-) stratified squamous epithelium, tracheobronchial remnants, abnormal mucous glands, a disorganised muscular coat and cartilage,[41-43] but ciliated epithelium has also been found.[42] Using immunohistochemical staining, RT-PCR and *in situ* hybridisation, the expression of specific proteins and genes in the TOF has been studied in both animals and humans. These include Thyroid Transcription Factor 1 (TTF-1), SHH and members of the BMP pathway.[43-48] Some data seem to support a respiratory origin of the TOF in humans, but the number of human TOFs examined was small, ranging from one to nine.

To gain more insight in the origin of the TOF, we aimed to examine and describe gene expression patterns in TOFs using an unbiased, whole-genome approach.

MATERIALS AND METHODS

Tissue morphology

To gain insight into the general morphology of the tissue of the TOFs, material from a group of patients with OA/TOF was used. After parental consent, tissue samples of the TOF were taken out during primary repair of the OA/TOF, as deemed appropriate by the operating surgeon. Samples were fixed in 10% buffered formaldehyde for two hours and after routine procedures embedded in paraffin.
Sections were deparaffinised in xylene and rehydrated in alcohol. Routine hematoxylin and eosin stainings and Elastica von Gieson stainings were done and the sections were evaluated for general structure of the tissue. In addition, immunohistochemical staining for TTF-1 was performed as follows. Endogenous peroxidase was blocked by 3% H_2O_2 in PBS for 20 minutes. Antigen retrieval was performed by heat induced epitope retrieval (HIER) in a Tris/EDTA buffer (pH 9.0) for 20 minutes. Staining was carried out using monoclonal mouse anti-human antibody (1:100; Neomarkers, Lab Vision Corporation, Fremont, CA, USA) for 30 minutes. Antigen-antibody complexes were visualised by a peroxidase-conjugated polymer DAB detection system (ChemMate DAKO Envision detection kit, Peroxidase/DAB, Rabbit/Mouse; Dako, Glostrup, Denmark).

Gene expression analysis

Patient samples

After parental informed consent was obtained, tissue samples of the TOF of a separate group of children with OA with a distal TOF were taken during primary repair of the OA/TOF. The technical feasibility of removing the tissue and the size of the tissue samples were determined by the operating surgeon, who had no involvement in the study. All samples were snap frozen in liquid nitrogen and stored at -80 °C until further processing.

Control samples

Control tissue (lung, trachea and oesophagus) was received from the tissue bank of the Erasmus MC. Control samples were taken from autopsies of children of 17 - 25 weeks gestational age who had died of causes not related to trachea, oesophagus or lung abnormalities and in whom there was no reason to assume any abnormalities of these organs. Only samples frozen less than 48 hours after death were used.

The protocol for this study was approved by the Medical Ethics Committee of the Erasmus MC.

RNA isolation and quality control

Patient and control samples were homogenized on ice in TRIzol reagent (Invitrogen life technologies, Carlsbad, CA, USA) and total RNA was isolated following the manufacturer's instructions. The only adjustment to the protocol was that the phase separation was repeated by adding 200 µl of 0.1% DEPC water to increase RNA yield.

RNA was purified using the Rneasy MinElute Cleanup kit (Qiagen, Valencia, CA, USA) and stored at -80°C until further processing. RNA concentrations and OD 260/280 nm ratios were measured using the NanoDrop® ND-1000 UV-VIS spectrophotometer (NanoDrop Technologies, Wilmington, USA).

Assessment of total RNA quality and purity was performed using the RNA 6000 Nano assay on the Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, USA).
Depending on the availability and/or quality of purified total RNA, cDNA was synthesized from 0.8 - 15 μg RNA using the GeneChip Expression 3'-Amplification Reagents One-Cycle cDNA Synthesis kit (Affymetrix, Santa Clara, CA, USA). Biotin-labelled cRNA synthesis, purification and fragmentation was performed according to standard conditions.
Fragmented biotinylated cRNA was subsequently hybridised onto Affymetrix Human Genome U133A Plus 2.0 microarray chips, which were scanned with the Affymetrix GeneChip Scanner 3000 or 7G.
The percentage of present calls, noise, background, and ratio of GAPDH 3' to 5' (< 1.5) all indicated a high quality of samples and overall comparability.

Data normalisation and analysis
Probe sets that were not present (according to Affymetrix MAS5.0 software) in any of the Genechips were omitted from further analysis. Raw intensities of the remaining probe sets of each chip were log2 transformed and raw expression values were Quantile normalized using quantile normalization. After normalization, the data were back-transformed to normal intensity values.
Data analysis was carried out using OmniViz software, version 3.6.0 (Omniviz, Inc., Maynard, MA, USA). For each probe set, the geometric mean of the hybridization intensities of all samples was calculated. The level of expression of each probe set was determined relative to this geometric mean and log 2 transformed (on a base 2 scale). The Pearson's correlation and visualization tool of OmniViz was used to study the result of unsupervised clustering op the samples (based on the log2 geometric mean values). In the correlation plot the samples are ordered by correlation. Samples with high positive correlations or high negative correlations are put into separate blocks and the cells are color-coded by correlation coefficient values.
Differentially expressed genes were identified using statistical analysis of microarrays (SAM analysis).

Gene network and pathway analysis
Significant genes identified in the SAM (Significance Analysis of Microarrays) analysis were entered into the Ingenuity Pathway Analysis (IPA) program (www.ingenuity.com). Each Affymetrix ID was mapped to its corresponding gene in the Ingenuity Pathway Knowledge Base. These genes, called Focus Genes, were overlaid onto a global molecular network developed from information contained in the Ingenuity Pathways Knowledge

Base. Networks of these Focus Genes were then algorithmically generated based on their connectivity.

LAP analysis

The Locally Adaptive statistical Procedure (LAP) was used to identify chromosomal regions with differential expression between cases and controls. This method combines expression data with structural information and accounts for variations in the distances between genes and in gene density.[49] LAP analysis was done in R, version 2.2.1.

Functions in R for implementing the LAP method were obtained from http://www.dpci.unipd.it/Bioeng/Publications/LAP.htm, the website of the university of Padua.

RESULTS

Tissue morphology

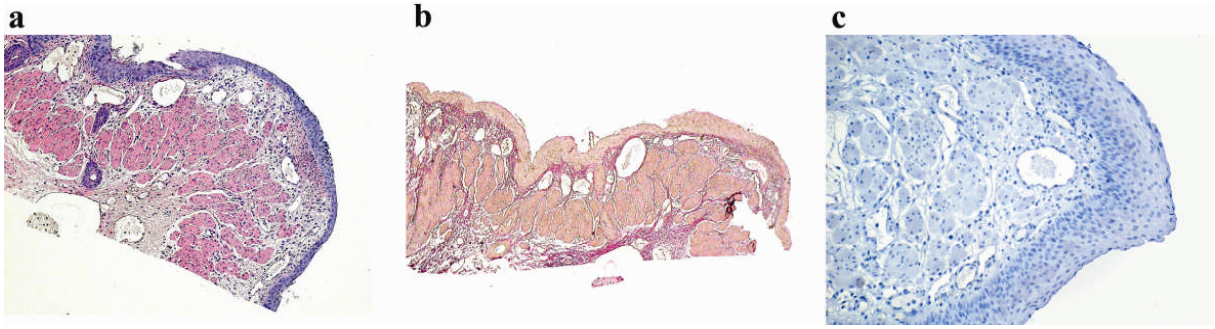
A total of 10 samples of the TOF from 8 patients were available. For one patient, there were three samples.

Hematoxylin and eosin and Elastica von Gieson stainings showed squamous epithelium in the TOFs. The mesenchyme showed connective tissue and a muscular layer that was disorganised in some TOF samples and less so in others. No cartilage was found (Figure 1a and b). TTF-1 stainings were negative in all TOF samples (Figure 1c).

Gene expression analysis

The total number of tissue samples collected during the study period was 41. Of these, 21 could be used for this study. Reasons for non-inclusion of samples were mostly low RNA yield or low RNA quality. Furthermore, three tracheal, three oesophageal and four lung samples were included as controls.

Figure 1 (*for colour figure see page 222*)

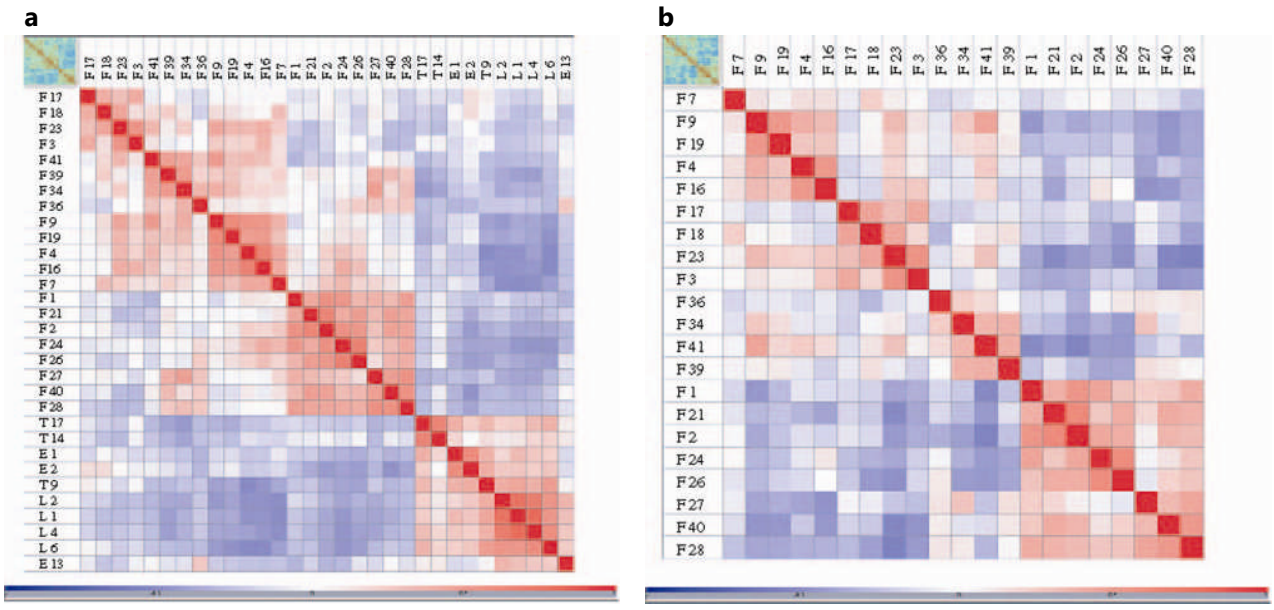


***a** Hematoxylin and eosin staining (10x), **b** Elastica von Gieson staining (5x) and **c** TTF-1 immunohistochemical staining (20x) of representative samples of the tracheo-oesophageal fistula (see text for details).*

General characteristics of the patients included in this study are shown in table 1.
Unsupervised clustering of all samples showed that the TOFs clearly differ from the control samples. When only those genes that showed at least a 2-fold change in expression level compared to the geometric mean in at least one sample were used for the unsupervised clustering, the difference between TOFs and controls was even more obvious (Figure 2a).

Within the group of TOFs a subdivision can be made on the basis of different gene expression patterns. Therefore, an unsupervised clustering was done within the TOF group, which showed an even more obvious distinction between the subgroups (one subgroup consisting of TOF numbers 1, 21, 2, 24, 26, 27, 40, 28; the other of the remaining TOFs) (Figure 2b).
The identified subgroups had no correlation with known clinical data, including gender, gestational age, birth weight, pregnancy characteristics, size of the tissue sample removed, time between birth and surgery, the presence of intra-uterine growth retardation and the presence of specific associated anomalies. In the larger of the two subgroups, there may be two or three further subgroups, but as the numbers in those groups were very small, we have not analysed these further.

Figure 2 (*for colour figure see page 223*)



*Correlation plots of all case and control samples (**a**) and of all TOF samples (**b**), including only those genes that showed at least a 2-fold change in expression level compared to the geometric mean in at least one sample. Samples are plotted against each other and the level of similarity of gene expression patterns is determined. Red: high similarity; Blue: low similarity (grading scale, see scale bar at bottom). F: TOF; T: trachea; E: oesophagus; L : lung. Numbers represent sample numbers.*

Table 1 Patient characteristics

Patient no.	Gender	GA (wk + d)	BW (g)	IUGR	Birth - surgery (d)	Outcome	Associated anomalies					
							Vertebral/Rib	Anal	Cardiac	Renal	Upper Limb	Other
1	F	37 + 5	2235	+	1	Alive	-	-	-	-	-	-
2	M	40 + 2	3595	-	0	Alive	+	-	-	-	-	A, B
3	F	34 + 6	1200	+	3	Deceased	-	-	-	-	-	CHARGE syndrome
4	F	36 + 2	2120	-	2	Alive	-	-	+	-	-	-
7	M	37 + 1	2865	-	2	Alive	-	-	-	+	-	-
9	M	37 + 2	3375	-	2	Alive	+	-	-	-	-	C
16	F	41 + 5	3170	-	2	Alive	+	-	-	-	-	D
17	M	38 + 4	3825	-	1	Alive	-	+	-	-	+	A, B, D, E
18	M	34 + 1	2060	-	3	Alive	-	-	+	-	-	-
19	M	31 + 2	1780	-	1	Alive	-	-	-	-	-	-
21	M	42 + 3	3800	-	1	Deceased	+	-	+	-	-	-
23	M	37 + 4	2640	-	2	Alive	-	-	+	-	-	-
24	F	41 + 0	3775	-	1	Alive	-	-	-	-	-	-
26	F	40 + 5	3570	-	1	Alive	-	-	-	+	-	-
27	M	40 + 4	3180	-	1	Alive	+	-	-	+	-	-
28	M	36 + 1	1800	+	1	Deceased	-	+	+	+	-	A, F
34	M	35 + 6	1780	+	2	Alive	-	-	+	+	-	A
36	M	42 + 0	3810	-	1	Alive	-	-	-	-	-	A, B
39	F	33 + 5	1750	-	1	Alive	-	-	-	+	-	B
40	M	40 + 1	3615	-	2	Alive	-	-	-	-	-	-
41	F	38 + 0	2800	-	2	Alive	-	-	+	-	+	-

F: female; M: male; GA: gestational age; wk: weeks; d: days; BW: birth weight; g: grams; IUGR: intra-uterine growth retardation; Birth-surgery: time between birth and surgery; A: single umbilical artery; B: dysmorphic features (mild in patient no.17, 36 and 39); C: cleft lip, jaw and palate; D: toe anomalies; E: hypospadias; F: duodenal atresia; CHARGE: Coloboma, Heart defects, Atresia of choanae, Retarded growth and development, Genital anomalies, Ear anomalies

TOFs compared to control samples

As expected, TOFs were by far the least similar to lung tissue, with 5496 probe ID sets showing a significant difference in expression level between TOFs and lung (Figure 3). The comparison between the TOFs and the other control samples, trachea and oesophagus, showed that the TOFs are more related to these structures. Therefore, we decided to focus on the comparison to trachea and oesophagus.

Comparison of the TOFs to the trachea samples revealed 2152 probe sets that were significantly different between cases and controls, representing 1417 mapped genes, of which 776 were eligible for building networks in Ingenuity.

The 5 most important networks all had the same significance score (37) and the same number of genes in the networks (35). Important functions of these networks in the frame of development of the TOF were cellular development, cell signalling and connective tissue development and function (Table 2).

Raising the stringency of our analysis to at least a 3-fold difference between the groups revealed 624 probe sets, representing 439 mapped genes, of which 282 were eligible for building networks.

On top of the list was one very specific network, of which an important function was cellular development. Genes involved in this network are shown in table 3.

SAM analysis of TOFs versus oesophagus showed 1288 probe sets that were significantly different between these two groups. These probe sets represented 909 mapped genes, of which 499 were eligible for generating networks.

Figure 3 Bar chart showing the number of probe ID sets on the gene expression array that showed a significant difference in expression level between the TOFs and the three different control groups (*for colour figure see page 224*)

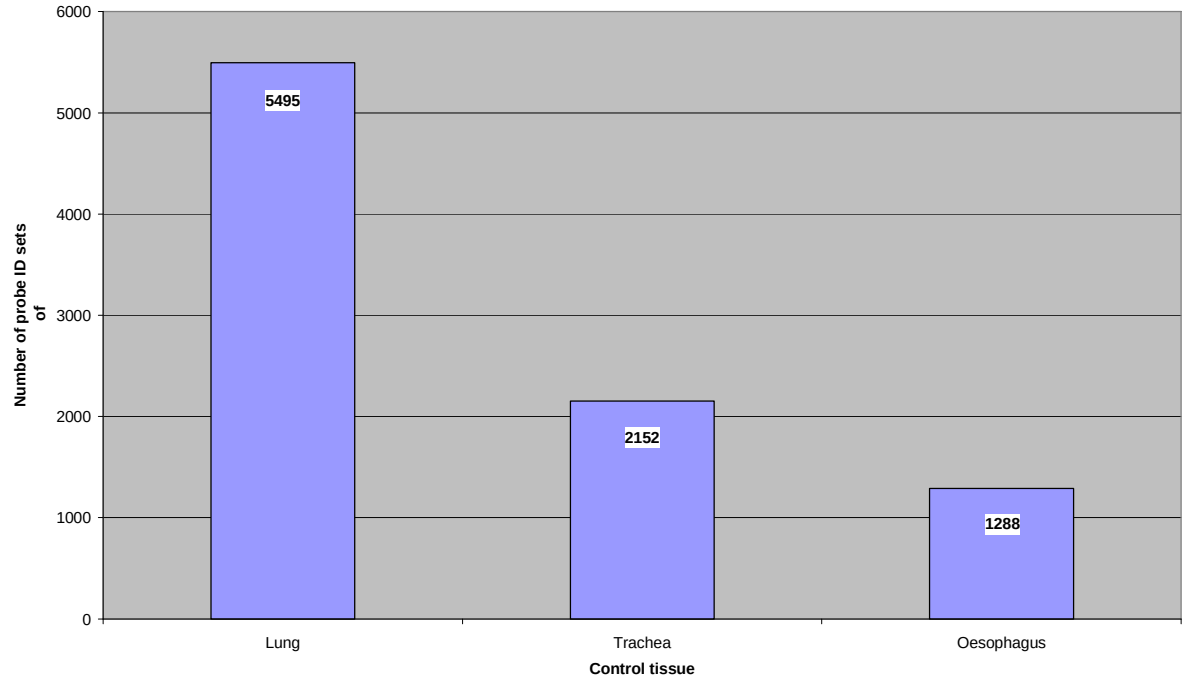


Table 2 Overview of genes in the five most important networks of the analysis of TOFs versus trachea and top functions of these networks

Downregulated genes	Upregulated genes	Top functions
ARID1B (includes EG:57492), CBFB, CCND3, CREBBP, DCX (includes EG:1641), GATA6, GPBP1, HES1, HEY1, HEY2, HOXD4, ID2, ID3, IGLL1, LEF1, MAML2, MST1, NR3C2, PAX5, PLAGL1, RBPSUH, SMAD1, SMARCA2, SPIB, SPP1, TCF4, TLE1, UBA52, UTX, VPREB1 (includes EG:7441)	ACTA2, ACTG2, MYH11, MYLK, TAGLN	Gene Expression Cellular Development Hematological System Development and Function
CBLB, CD247, CD3D, CD3E, CD3G, CD79A, CD79B, CD8A, CLEC2D (includes EG:29121), EBF, FYN, GLTSCR2, IGHM, IL7R, ITK, KLRB1, LAT, LCK, MS4A1, PAG1, PDGFRB, PLCG1, POU2AF1, PTEN, PTK2, PTPRA (includes EG:5786), PTPRCAP, RASA1, SIT1, TCF3, TRA@, TRAT1, UNC5C, WDR48, ZAP70	-	Cell Signaling Post-Translational Modification Cellular Development
ADAM12, AGC1, APAF1, ATM, BCL2, BCLAF1, BIRC2, BIRC4, CAMK4, CASP2, CDKN1C, CHST1, CXCL12, GPC3, IGF2, IGFBP3, IGFBP5, MICAL1, MMP2, MMP9, MMP14, MMP16, NALP1, PLAG1, POT1, PPP2R2B, PROX1, RAB8B, RAD17, RAD9A, TERF1, TERF2IP, THBS2, TNKS2, VIM	-	Connective Tissue Development and Function Skeletal and Muscular System Development and Function Cancer
ABCA1, ABCG1, ANP32A, APOA1, APOA2, APOC1, APOE, BGN, F2R, GHR, GZMA, HBE1, HBZ, HMGB2, LAMB1, LAMC3, LECT1, LPL, MFAP2, NID2, NR1H3, NR2F1, NR2F2, NRP1, PROCR, PTN, RECK, RORC, SEMA3D, SET, SP3, SP100, SPARC, UTRN, VEGF	-	Cardiovascular Disease Metabolic Disease Lipid Metabolism
AHR, AIP, ANP32B, CDKN1B, COL1A1, COL1A2, COL2A1, COL3A1, CRSP3, CRSP7, DCN, ETS1, GABARAP, GATA3, ITGA11, ITGAL, ITGB2, LMO2, LTB, MAF, MED6, MRC2, OPRK1, P4HA1, PRKD2, PRRX1, RBL2, SATB1, SFRP1, SOX5, TAL1, TCF12, TCF7L2, XPO1, ZNFN1A1	-	Gene Expression Cellular Development Hematological System Development and Function

Table 3 Genes from most significant network of the analysis of TOFs versus trachea, including only those genes showing a 3 fold or higher difference in expression level (see text for details)

Gene	Description	Fold change
Kinases		
LCK	lymphocyte-specific protein tyrosine kinase	↓ 8.02
PAK3	p21 (CDKN1A)-activated kinase 3	↓ 3.98
ZAP70	zeta-chain (TCR) associated protein kinase 70kDa	↓ 5.78
Other		
IGHM	immunoglobulin heavy constant mu	↓ 44.17
IGJ	immunoglobulin J polypeptide, linker protein for immunoglobulin alpha and mu polypeptides	↓ 25.85
IGKC	immunoglobulin kappa constant	↓ 46.79
IGL@	immunoglobulin lambda locus	↓ 42.95
IGLL1	immunoglobulin lambda-like polypeptide 1	↓ 3.83
LY9	lymphocyte antigen 9	↓ 4.61
MBP	myelin basic protein	↓ 7.21
NRCAM	neuronal cell adhesion molecule	↓ 3.08
PLP1	proteolipid protein 1	↓ 5.30
PTPRCAP	protein tyrosine phosphatase, receptor type, C-associated protein	↓ 8.31
SH2D1A	SH2 domain protein 1A	↓ 5.95
SIT1	signaling threshold regulating transmembrane adaptor 1	↓ 4.33
TRA@	T cell receptor alpha locus	↓ 9.62
TRAT1	T cell receptor associated transmembrane adaptor 1	↓ 4.10
TRPM7	transient receptor potential cation channel, subfamily M, member 7	↓ 3.10
Phosphatases		
PTPRC	protein tyrosine phosphatase, receptor type, C	↓ 4.34
PTPRD	protein tyrosine phosphatase, receptor type, D	↓ 5.53
Transcription regulators		
EBF1	early B-cell factor 1	↓ 3.02
LEF1	lymphoid enhancer-binding factor 1	↓ 3.33
PAX5	paired box gene 5 (B-cell lineage specific activator)	↓ 3.34
POU2AF1	POU domain, class 2, associating factor 1	↓ 4.80
SATB1	special AT-rich sequence binding protein 1	↓ 3.07
SOX4	SRY (sex determining region Y)-box 4	↓ 4.74
Transmembrane proteins		
CD247	CD247 molecule	↓ 7.08
CD3D	CD3d molecule, delta (CD3-TCR complex)	↓ 7.98
CD3E	CD3e molecule, epsilon (CD3-TCR complex)	↓ 3.12
CD3G	CD3g molecule, gamma (CD3-TCR complex)	↓ 5.72
CD38	CD38 molecule	↓ 3.02
CD79A	CD79a molecule, immunoglobulin-associated alpha	↓ 4.93
CD8A	CD8a molecule	↓ 3.02
CLEC2D	C-type lectin domain family 2, member D	↓ 3.92
KLRB1	killer cell lectin-like receptor subfamily B, member 1	↓ 4.70

↓ *Expression of gene significantly downregulated in TOF compared to trachea*
↑ *Expression of gene significantly upregulated in TOF compared to trachea*

The three most important networks had a number of interesting functions, including cell-to-cell signalling and interaction, gene expression, tissue development, cell morphology and cellular assembly and organization (Table 4).
Further narrowing down the selection of genes in the analysis by only including those genes with a 3-fold or more difference in expression level between cases and controls gave 456 probe sets, representing 321 mapped genes, of which 198 were eligible for building networks. One network, involved in tissue morphology and tissue development (among other functions), came out far on top of the list. Genes in this network are shown in table 5.

Absent/present analysis
Even though the biological variation of the fistula samples is rather large, we reasoned that they could have a resemblance of their gene signature. Therefore, we searched for characteristics that all 21 TOFs had in common. At the start of the analysis, all probe sets were characterised per sample to be "present", "marginal" or "absent" and for this analysis, the "marginal" probe sets were left out.

Firstly, a total of 14,662 probe sets were "present" in all 21 TOFs and this set was used for the SAM analyses. Analysis of TOFs versus lung revealed 2558 probe sets that had a significantly decreased expression level in TOFs and 796 probe sets that were significantly increased. In TOFs versus trachea, 1466 probe sets were significantly decreased in TOFs and 113 were significantly increased. In TOFs versus oesophagus, 879 probe sets were significantly lower and 21 were significantly higher in TOFs. The intersection of these analyses revealed 472 probe sets to be significantly decreased and 20 to be significantly increased in TOFs compared to lung as well as trachea as well as oesophagus (Figure 4a). Of note is, that of the 21 genes that had a significantly increased expression in the TOFs compared to the oesophagus, 20 were also in the intersection.

Secondly, The 2663 probe sets that were "absent" in all 21 TOFs were subjected to the SAM analysis, revealing that 751 probe sets were significantly decreased in TOFs versus lung, 466 in TOFs versus trachea and 309 in TOFs versus oesophagus. The intersection of these three analyses showed 94 probe sets that were significantly decreased in TOFs versus all controls (Figure 4b).

The list of 586 probe sets that were found in the intersections described above (472 present in all TOFs and significantly decreased in TOFs versus all controls, 20 present in all TOFs and significantly increased in TOFs versus all controls and 94 absent in all TOFs and significantly decreased in TOFs versus all controls) were analysed with Ingenuity. This set of 586 probe IDs represented 436 mapped genes, of which 251 were eligible for generating networks.
The top network found had a much higher score and many more of the genes included than the other networks. Genes in this network are shown in table 6. The most interesting general function in which this network played a role was, again, cellular development.

Table 4 Overview of genes in the three most important networks of the analysis of TOFs versus oesophagus and top functions of these networks

Downregulated genes	Upregulated genes	Top functions
AHR, ANP32A, BRD8, CKM, CREBBP, CRSP3, DACH1, DDX17, GPBP1, IGHM, IGKC, IGL@, MAF, MEF2C, MST1, MYF5, NCOA1, NCOA2, NEDD4L, NR2F1, NR3C2, PLAGL1, PPARG, RPS6KA3 (includes EG:6197), SCNN1B, SCNN1G, TAF7, TCF21, THRAP1, TPM2, TRIM24, TRIM33, VGLL4, XPO1	TAGLN	Genetic Disorder Metabolic Disease Gene Expression
CCL5, CCR7, CD55, CDH11, CSPG2, CTNNB1, CXADR, CXCL12, CXCR4, DPP4, ERBB2IP, FBLN1, FGG, FYN, HIST2H2AA3, HIST2H2BE, HNRPA1, HNRPD, HSPA1A, HSPD1, LGALS3, LUM, MKNK2, MMP14, MS4A1, PDZD2, PIK3CA, PKN2, PTPRA (includes EG:5786), PTPRD, PTPRF, RPS25, TCF4, TMSL8, XRN1	-	Cell-To-Cell Signaling and Interaction Tissue Development Cancer
ACTA1, ACTN2, ADAM12, CAPN3, CAPN7, COL1A2, COL3A1, DNMT3A, DRD1, EED, GDF15, GPC3, HSP90B1, IGF2, IGF2BP3, KBTBD10, KCNQ1OT1, MAT2B, MSH6, MYH7, MYOZ1, NEB, NUP153, P4HA1, RAB8B, RBBP4, SLN (includes EG:6588), SMARCC1, SP3, SUZ12, TCF12, TTN, VIM	ACTA2, ACTG2	Cell Morphology Cellular Assembly and Organization Skeletal and Muscular System Development and Function

Table 5 Genes from the most significant network from the analysis of TOFs versus oesophagus, including only those genes showing a 3 fold or higher change in expression level (see text for details)

Gene	Description	Fold change
Growth factors		
IGF2	insulin-like growth factor 2 (somatomedin A)	↓ 9.01
Ion channels		
RYR1	ryanodine receptor 1 (skeletal)	↓ 5.69
Kinases		
CKM	creatine kinase, muscle	↓ 25.55
Others		
KBTBD10	kelch repeat and BTB (POZ) domain containing 10	↓ 19.80
POSTN	periostin, osteoblast specific factor	↓ 3.07
Others – muscle related proteins		
ACTA1	actin, alpha 1, skeletal muscle	↓ 13.58
ACTC	actin, alpha, cardiac muscle 1	↓ 3.58
ACTG2	actin, gamma 2, smooth muscle, enteric	↓ 4.88
BIN1	bridging integrator 1	↓ 3.60
CASQ1	calsequestrin 1 (fast-twitch, skeletal muscle)	↓ 4.26
CSRP3	cysteine and glycine-rich protein 3 (cardiac LIM protein)	↓ 9.87
LDB3	LIM domain binding 3	↓ 7.06
LRRC7	leucine rich repeat containing 7	↓ 3.48
MYH3	myosin, heavy chain 3, skeletal muscle, embryonic	↓ 32.06
MYL4	myosin, light chain 4, alkali; atrial, embryonic	↓ 6.65
MYLPF	fast skeletal myosin light chain 2	↓ 53.14
MYOZ1	myozenin 1	↓ 7.79
MYOZ2	myozenin 2	↓ 11.35
NEB	nebulin	↓ 13.22
TNNC2	troponin C type 2 (fast)	↓ 47.07
TNNI1	troponin I type 1 (skeletal, slow)	↓ 10.69
TNNI2	troponin I type 2 (skeletal, fast)	↓ 52.71
TNNT2	troponin T type 2 (cardiac)	↓ 3.35
TNNT3	troponin T type 3 (skeletal, fast)	↓ 6.40
TPM2	tropomyosin 2 (beta)	↓ 13.15
TRDN	triadin	↓ 12.70
Peptidases		
CAPN3	calpain 3, (p94)	↓ 16.67
CAPN6	calpain 6	↓ 3.68
TTN	titin	↓ 24.79
Transcription regulators		
ACTN2	actinin, alpha 2	↓ 16.24
HEY1	hairy/enhancer-of-split related with YRPW motif 1	↓ 3.71
MYF5	myogenic factor 5	↓ 6.88
MYF6	myogenic factor 6 (herculin)	↓ 3.40
MYOD1	myogenic differentiation 1	↓ 3.94
PLAG1	pleiomorphic adenoma gene 1	↓ 3.24

↓ *Expression of gene significantly downregulated in TOF compared to oesophagus*
↑ *Expression of gene significantly upregulated in TOF compared to oesophagus*

Figure 4 (*for colour figure see page 225*)

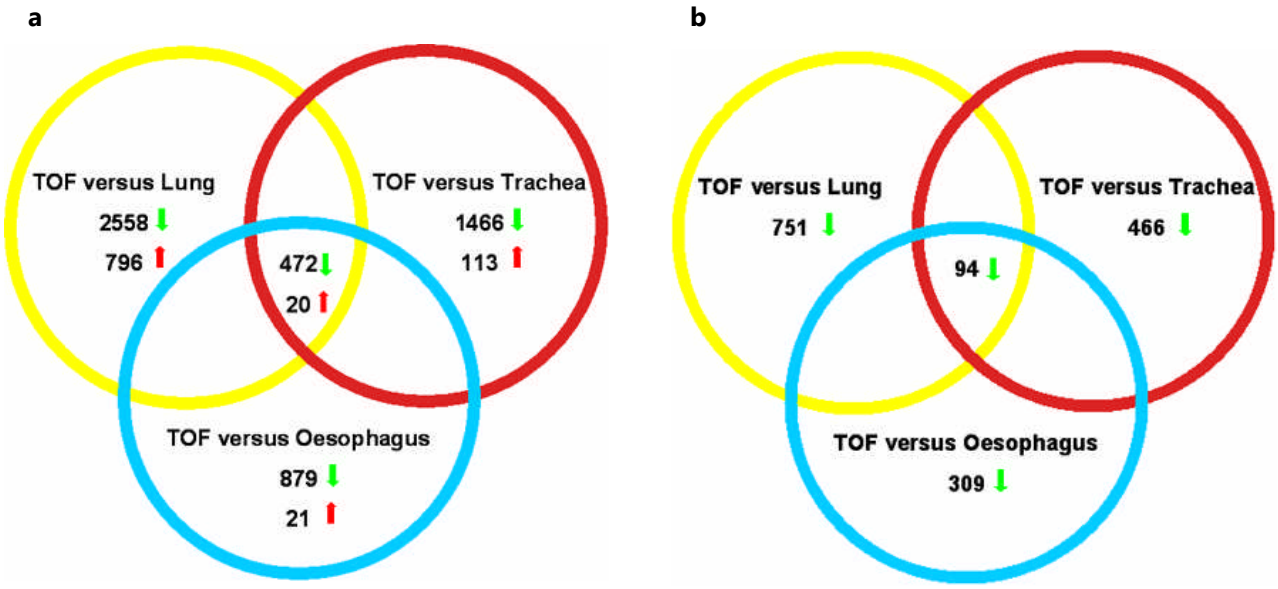


*Diagram showing the results of the SAM analyses of TOFs versus the three control groups including only those genes that were present in all 21 TOFs and the intersection of these analyses (**a**) and including only those genes that were absent in all 21 TOFs and the intersection of these analyses (**b**).*

LAP analysis

In order to discriminate chromosomal regions that contained clusters of genes that were all up- or downregulated in TOFs versus control samples, we performed a LAP analysis. This showed a relatively large number of chromosomal loci with genes that were significantly up- or downregulated in TOFs compared to trachea and also in TOFs compared to oesophagus ($P < 0.05$) (figures 5a and b). Again, the differences between TOFs and lung were so large (data not shown) that we decided to focus on trachea and oesophagus as control groups.

Lowering the significance threshold limited the number of significantly changed loci to three and six at a P-value of < 0.02 in the analysis of TOFs versus trachea and TOFs versus oesophagus, respectively (data not shown).

At the most stringent significance threshold of $P < 0.01$, the number of significant loci was two (on chromosome 13q and on chromosome 2q) in the comparison of TOFs versus trachea and two as well (on chromosome 13q and on chromosome 5q) in the comparison of TOFs versus oesophagus (data not shown). All loci showed decreased expression in the TOFs compared to the controls. The only gene that showed a significant difference in both the comparison with oesophagus and the comparison with trachea was *Foxo1a*, located on chromosome 13q14.1.

Table 6 Genes from most significant network of the analysis of TOFs versus all control groups, only based on those probe ID sets that were absent or present in all TOFs (see text for details)

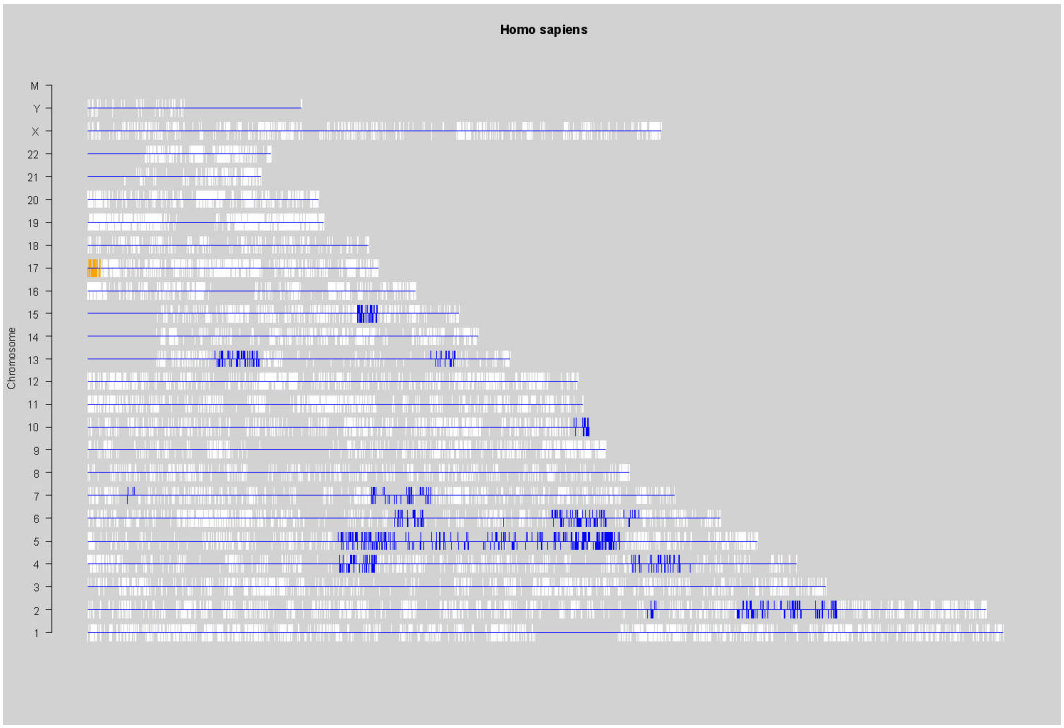
Gene	Description	Change *
Cytokines		
CXCL12	chemokine (C-X-C motif) ligand 12 (stromal cell-derived factor 1)	↓
Enzymes		
MAT2B	methionine adenosyltransferase II, beta	↓
MICAL1	microtubule associated monoxygenase, calponin and LIM domain containing 1	↓
RAB8B	RAB8B, member RAS oncogene family	↓
VCL	vinculin	↑
G-protein coupled receptors		
CXCR4	chemokine (C-X-C motif) receptor 4	↓
Growth factors		
VEGFA	vascular endothelial growth factor A	↓
Kinases		
FYN	FYN oncogene related to SRC, FGR, YES	↓
PDK1	pyruvate dehydrogenase kinase, isozyme 1	↓
PTK2	PTK2 protein tyrosine kinase 2	↓
Ligand-dependent nuclear receptors		
NR2F1	nuclear receptor subfamily 2, group F, member 1	↓
RORC	RAR-related orphan receptor C	↓
Others		
ACTA2	actin, alpha 2, smooth muscle, aorta	↑
ACTG2	actin, gamma 2, smooth muscle, enteric	↑
CBLB	Cas-Br-M (murine) ecotropic retroviral transforming sequence b	↓

CDKN1C	cyclin-dependent kinase inhibitor 1C (p57, Kip2)	↓
COL1A2	collagen, type I, alpha 2	↓
CSPG2	chondroitin sulfate proteoglycan 2 (versican)	↓
LUM	lumican	↓
MYH11	myosin, heavy chain 11, smooth muscle	↑
NEDD9	neural precursor cell expressed, developmentally down-regulated 9	↓
TAGLN	transgelin	↑
VIM	Vimentin	↓
Peptidases		
DPP4	dipeptidyl-peptidase 4 (CD26, adenosine deaminase complexing protein 2)	↓
MMP2	matrix metallopeptidase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase)	↓
MMP14	matrix metallopeptidase 14 (membrane-inserted)	↓
Transcription regulators		
HES1	hairy and enhancer of split 1, (Drosophila)	↓
MAML2	mastermind-like 2 (Drosophila)	↓
PLAG1	pleiomorphic adenoma gene 1	↓
SP3	Sp3 transcription factor	↓
TCF3	transcription factor 3 (E2A immunoglobulin enhancer binding factors E12/E47)	↓
TCF4	transcription factor 4	↓
Transmembrane receptors		
CD247	CD247 molecule	↓
CD36	CD36 molecule (thrombospondin receptor)	↓
IL7R	interleukin 7 receptor	↓

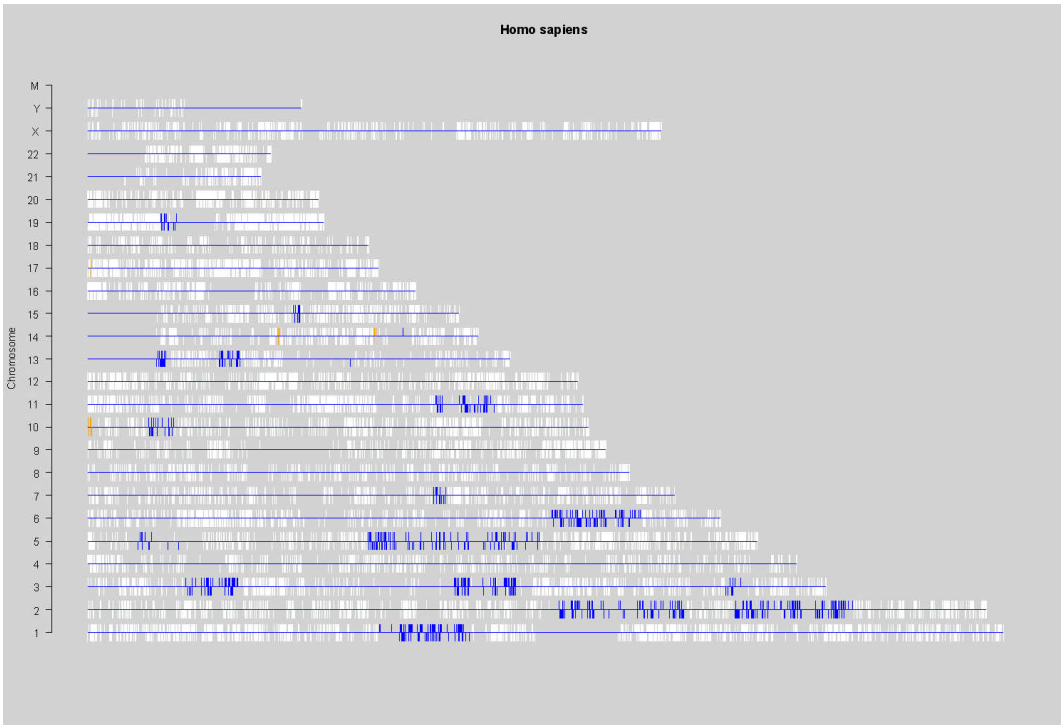
↓ Expression of gene significantly downregulated in TOF compared to all control groups
↑ Expression of gene significantly upregulated in TOF compared to all control groups
** As TOFs are compared to the individual control groups first and results then combined, fold changes cannot be calculated for this analys*

Figure 5 (*for colour figure see page 226*)

a



b



*Results of the LAP analysis showing significantly up- (orange) or downregulated (blue) chromosomal loci in TOFs compared to trachea (**a**) and oesophagus (**b**). A cut-off P-value of 0.05 was used for significance. Chromosome numbers are shown on the left-hand side of the picture. Chromosomes are shown as bars with the p-arm on the left-hand side and the q-arm on the right hand side. White vertical lines depict non-significantly different areas.*

DISCUSSION

This study is the first to describe the analysis of gene expression patterns in human tracheo-oesophageal fistulas compared to normal tracheal, oesophageal and lung tissue. The whole-genome approach allowed us to look open-mindedly and unbiased at genes and functions that may have a role in the development of this severe congenital anomaly, of which the aetiology is still largely unknown. The study was set up to create a starting point for further, more detailed studies.

Unsupervised clustering analysis revealed that the TOFs all clustered separately from the controls. This means that the TOFs represent a specific type of tissue and that they share more characteristics with each other on the level of gene expression than with the control tissues.
Within the total group of 21 TOFs, at least two subgroups could be appreciated. There was a very distinct and homogenous subgroup of 8 samples and a larger, less well-defined subgroup of 13 samples. We have not been able to find significant associations between these subgroups and any known clinical data. However, these subgroups may represent different aetiologies affecting different developmental pathways leading to the same phenotype at birth. Alternatively, one aetiological factor may be able to cause disturbances in different developmental pathways all leading up to the development of a tracheo-oesophageal fistula. Further analysis of the differences between the TOF subgroups may shed more light on this.

Looking at the number of differentially expressed genes, the TOFs seem to be most similar to oesophagus and least similar to lung. This is also the case when one looks at the results of the absent/present analysis (Figure 4a and 4b).

The group of TOFs is not homogenous and the samples were not all the same size and larger tissue samples may have contained more "distal oesophageal" tissue than smaller ones. In addition, not all TOFs may have the same aetiology and this may be visible in their gene expression patterns. We included as many TOFs as possible to try to smoothen out the largest differences between individual samples.
To filter out the expected (biological) variation between the TOF samples, we tried to make our case group as large as possible. We hoped that this approach would eventually result in a set of genes that probably is characteristic for the TOFs. We expected these genes to be at the periphery of a network and hoped that they would lead to (a) common upstream target(s). Therefore, we decided to look at genes that were either present or absent in all TOFs and compare their expression levels in TOFs with those in controls. Although 21 TOF samples is still a relatively small group, especially compared to the large sample sizes in oncology studies, sample collection took around four years, underlining the difficulty in obtaining this material. Other studies involving human TOF material also report small numbers, the largest one consisting of nine patients.[43,46,48]

The selection of the most suitable control tissues was a major issue in this study. It is very difficult to obtain controls that are as close to the affected tissue as possible. Organs emerging from the same structure (foregut), namely the trachea and the oesophagus, were used as controls. As an extra foregut-derived control, we used lung tissue. Our attempt to find differences was rewarded by the fact that the TOFs do have a specific expression profile, which separates them from the trachea, oesophagus and lung. However, the need to use different types of tissue as controls results in the generation of gene expression profiles that are already quite distant from the TOFs, as opposed to, for example, oncology studies that use tumour tissue versus normal tissue from the same organ. These gene expression profiles may not have any aetiological role, but may simply represent differences between tissues. Therefore, it was to be expected that we would find many differentially expressed genes.

All TOFs studied were term or near term. However, the formation of the TOF takes place in early gestation. The gene expression patterns of TOFs surgically removed at or close to term may not represent the pattern of expression during development of the TOF and thus may not provide accurate information about genes that play a possible role in this faulty development. As it is virtually impossible to obtain either case or control material from around the time the TOF develops, we used a group of samples collected from terminations of pregnancy or spontaneous abortions as early as possible in pregnancy, which in this case was between 17 + 1 weeks and 25 + 5 weeks. Unfortunately, we were unable to retrieve sufficient and qualitatively good material from normal term neonates to use as a control group that was matched for gestational age.
It is difficult to say what the effects of this are on the results of the study. On the one hand, distinct differences in gene expression patterns have been described in mouse tissue from different developmental stages.[50] On the other hand, despite these differences, tissue samples from one organ may still show a characteristic gene expression pattern for that organ and may therefore still cluster together. In the latter case, comparing the TOFs to preterm control samples would give slightly different results than comparing them to term control samples, but the most important differences between the tissue types would still be found, as these differences are larger than those between samples taken from the same tissue type at different gestational ages. Unfortunately, we cannot draw definite conclusions about this at present, as too little is known about gene expression patterns of these human tissues at different gestational ages. Future studies will have to shed more light on this issue.

Tissue samples contained a mixed cell population and percentages of cell types (e.g.mesenchymal or epithelial cells) may have varied between samples. Separating the different cell types was, however, technically not feasible.

In order to obtain a more specific list of genes, we applied additional, more stringent criteria, such as only looking at genes showing a relatively large difference (3 fold change or more) in expression level compared to the controls. This approach suggests that genes

with a large difference in expression level also represent those genes with an important role in underlying developmental processes, which may not always be the case. However, we hoped that it would reveal downstream targets of these regulators, and in this way we would be able to have a more focussed approach.

This study was set up as a pilot study to gain some insight into the thus far very unclear molecular pathways that have a role in the development of OA/TOF. Our results show a large number of differentially expressed genes between TOFs and the different control tissues. The software programs we used allow us to interpret these differences in terms of general networks and functions. Many different functions came out of the analysis. In itself, this is not surprising, as we studied different tissue types. Also, many processes were found that might be involved in development in general, such as tissue morphology and cellular development. The software programs do not give us definite answers at present about genes or pathways leading to the development of the TOF. Many further studies will have to be done to give us more insight in these matters and the interpretation of the processes found should be left until more studies have been done.
The LAP analysis revealed a number of chromosomal loci with significant differential expression between TOFs and controls. Our group recently published a review of chromosomal anomalies described in patients with OA/TOF in the literature.[51] The loci that were found in the analysis using the most stringent criterion of $P < 0.01$ showed no overlap with these previously published anomalies. At a significance level of $P < 0.05$, the analysis of TOFs versus trachea showed the terminal end of chromosome 10q to be downregulated in the TOFs (Figure 5a). This area remained significant at $P < 0.031$, but not at $P < 0.02$ (data not shown). There is one report in the literature of a deletion of 10q25.3-qter in a patient with OA/TOF and multiple other congenital anomalies.[52] However, deletions of the end of the long arm of chromosome 10 have been described relatively frequently in the literature and to the best of our knowledge, none of the other cases displayed OA/TOF.[53-55] Therefore, it is at present unclear if there is an aetiological link between this chromosomal area and the development of OA/TOF. Downregulation of an area on chromosome 5q32 was found at a significance level of $P < 0.05$, but lost at more stringent levels (Figure 5a).

In the analysis of TOFs versus oesophagus using $P < 0.05$ (and also still significant at $P < 0.031$, data not shown), a downregulation of part of chromosome 3p was seen in the TOFs (Figure 5b). To the best of our knowledge, deletions of neither 5q32 nor of 3p have been described in combination with OA/TOF.

The database of the Erasmus MC - Sophia Children's Hospital in Rotterdam, the Netherlands, contains clinical data on all 255 patients with OA/TOF treated in this hospital since 1988. Karyotypes are available for 170 of these patients. 160 of these were normal, 9 showed various trisomies and 1 had a balanced translocation. However, normal karyotyping may not detect small changes. Therefore, if candidate regions are found and

confirmed in the future, re-evaluation of these patients with more detailed techniques may be warranted.
The only gene that was found in the analysis of TOFs versus trachea as well as in the analysis of TOFs versus oesophagus was *Foxo1a*. This gene has been described to be a negative regulator of insulin sensitivity in liver, adipocytes and beta-cells and to be involved in the negative regulation of skeletal muscle mass.[56,57] However, it is too early to say that this gene plays a role in the development of OA/TOF. Rather than having a role in the faulty foregut development, *Foxo1a* may also act as a classifying gene. As described below, the results of these analyses will first have to be confirmed.

The regions found in the LAP analysis may represent genomic imbalances, but this will have to be confirmed in further studies. However, it is also possible that in development, "blocks" of genes adjacent to each other are switched on or off during development by processes such as methylation. These epigenetic mechanisms may also have a role in the pathogenesis of the TOF and this is, again, an interesting focus for future research.

As with all microarray experiments, the results found have to be confirmed using other techniques. At present, it is too early to do these confirmation experiments, as they have to be done on a gene-by-gene basis, a process for which a list of target genes has to be available. Should a number of target genes be found, they will have to be tested using Q-PCR or immunohistochemistry on tissue samples from TOFs from a separate group of patients to confirm the results found in this study.

Different types of TOFs, such as distal, proximal or H-type TOFs may represent different aetiologies. As the latter two types of TOFs are very rare, much larger studies would be needed to draw conclusions about this.

This study represents a first attempt at characterisation of gene expression patterns in the tracheo-oesophageal fistula. A definitive answer regarding the aetiology of pathogenesis of OA/TOF can not be provided by this research.

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A new missense *MYCN* mutation in a child with Feingold syndrome

Chapter 7

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ABSTRACT

A boy with clinical characteristics of Feingold syndrome is described, in whom a *de novo* c.1226C>T mutation of the *MYCN* gene was found. This mutation is predicted to result in the substitution of a proline with a leucine at position 409 (p.P409L). This is the first report of this specific missense mutation in *MYCN*. The amino acid that is changed is situated in the loop of the helix-loop-helix domain. This mutation is predicted to be pathogenic. Several family members displayed minor anomalies that could also be part of Feingold syndrome. However, no mutation was found in these relatives. In addition, the patient had behavioural problems, of which it is not certain if they should be considered part of the syndrome.

INTRODUCTION

Feingold syndrome, also called ODED (oculo-digito-esophago-duodenal), MODED (microcephaly-oculo-digito-esophageal-duodenal) or MMT (microcephaly-mesobrachy-phalangy-tracheo-oesophageal fistula) syndrome, is an autosomal dominant syndrome with variable expression (OMIM 164280). Patients display digital anomalies, microcephaly, learning disability, short palpebral fissures and anomalies of the digestive system. The latter symptom is present in around 40% of cases. Of these anomalies, oesophageal atresia and/or tracheo-oesophageal fistula (OA/TOF) is the most commonly occurring malformation, present in 25% of patients. Duodenal atresia occurs in 16% of cases.[1-4] Microcephaly is present in 86% of cases.[3] Digital anomalies are mostly clinodactyly and shortness of the 2nd and 5th fingers with hypoplasia of the middle phalanx, and syndactyly of the toes (2 and 3, or 4 and 5).[5] Short stature and vertebral, renal and cardiac anomalies have also been described.[3,6] Heterozygous mutations of the *MYCN* gene on chromosome 2p24.1 have been shown to be the cause of Feingold syndrome.[1]

Besides *MYCN*, three genes have been reported to be involved in the aetiology of syndromic OA/TOF, namely *CHD7* (CHARGE syndrome, OMIM 214800), *SOX2* (AEG-syndrome) and *MID1* (Opitz syndrome, OMIM 300000) (for review see ref. 7-9).

The phenotype of Feingold syndrome partially overlaps with that of the VACTERL (*V*ertebral, *A*nal, *C*ardiac, *T*racheo-o*E*sophageal, *R*enal, *L*imb) association. It is therefore of interest that *MYCN* has been shown to be a target of Sonic hedgehog (Shh) signalling, at least in mouse cerebellum.[10] Knockout mice for *Shh* display a phenotype remarkably similar to that of the VACTERL association.[11,12] So far, mutations of *SHH* have not been associated with the VACTERL association in humans.[13]

In this paper, we performed *MYCN* mutation analysis in a family with clinical characteristics suggestive of Feingold syndrome.

CLINICAL REPORT

The patient was a boy, born by vaginal forceps delivery at a gestational age of 39 weeks; his birth weight was 2970 grams (approximately –1 SD). He was the first child of a 29-year-old mother and a 27-year-old father, who were non-consanguineous. The pregnancy had been uneventful, except for a fever around the 14th week. Apgar scores were 9 and 10 after one and five minutes, respectively. Soon after birth, oesophageal atresia (OA) with a distal tracheo-oesophageal fistula (TOF) was diagnosed. On the second day of life, he had surgery to ligate the fistula and to restore oesophageal continuity. Clinical examination revealed a cutaneous syndactyly of the second and third toes bilaterally and a head circumference of 33 centimetres (just above -2 SD). There were no cardiac, renal, vertebral, genital or anal anomalies. Cranial ultrasound study

showed no structural brain abnormalities. At that time, no obvious facial dysmorphisms were noted, except for a small helix of both ears. Standard cytogenetic analysis showed a normal 46,XY male karyotype and no syndrome diagnosis was made.

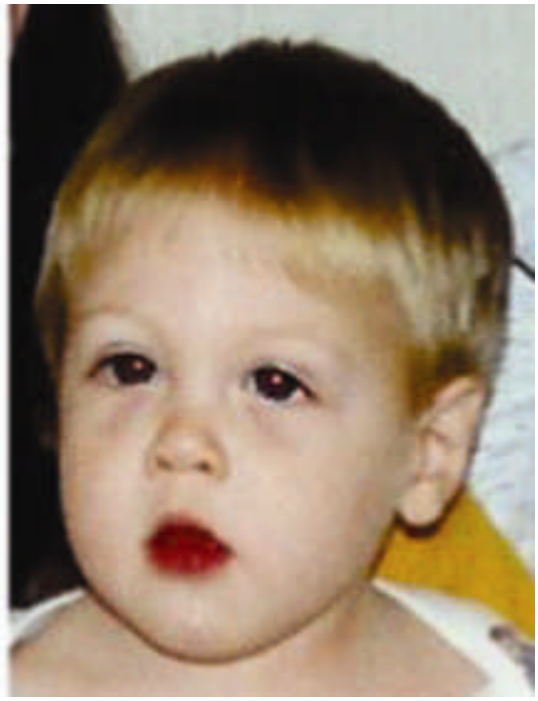
When the patient was almost 1 year of age, he and his family were referred for genetic counselling. At that time, his weight was 8715 grams (just below -1 SD) and his height was 72.5 centimetres (-1 SD). The patient now showed downslanting palpebral fissures, a flat nasal bridge, telecanthus and bilateral epicanthus, and a small mouth with a thin upper lip (Figure 1a). His head circumference was 43.5 centimetres (just above –2.5 SD). There was brachydactyly and clinodactyly of the fifth finger bilaterally (Figure 1b). He had brittle nails and a cutaneous syndactyly of the second and third toes bilaterally (more so on the left than on the right), as was noted before (Figure 1c). His behaviour was appropriate for his age.
Family history revealed that the father (Figure 1d and e) and the father's sister had short fingers and toes, but a normal head circumference: 57 cm (-0.5 SD) and 55 cm (0 SD), respectively. Maternal head circumference was also normal: 57.5 cm (1 to 1.5 SD). There was no family history of learning disability or developmental delay. The paternal grandmother had short toes and syndactyly of the second and third toes.

X-ray images of hands and feet showed that the patient had a short middle phalanx of both fifth fingers with clinodactyly and a relative elongation of the metacarpals and proximal phalanges. The distal phalanges were normal (Figure 2). His feet showed short distal phalanges and no ossification of the middle phalanges, except for the right second toe. The mother had normal hands and brachymesophalangy of the feet. The hands of both the father and the paternal aunt showed short middle and distal phalanges and normal metacarpals and proximal phalanges. They both had brachymesophalangy of the feet and the aunt in addition had brachytelephalangy of the feet.

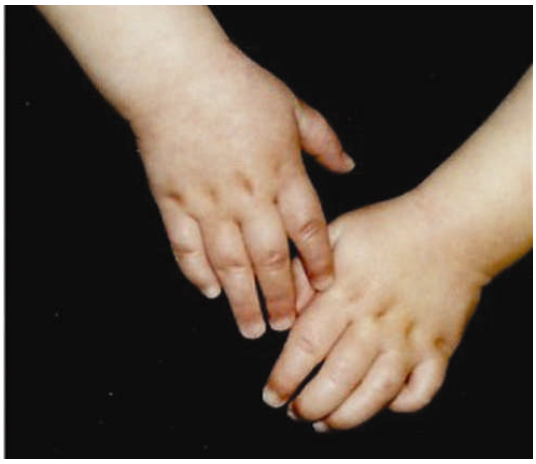
Although the patient did not have all typical characteristics, Feingold syndrome was suspected, on the grounds of OA/TOF and the relatively small head. The limb anomalies seen in the father, the father’s sister and his mother were interpreted as possibly representing variable expression of this syndrome. Alternatively, the patient, his father and his aunt might have a familial form of short toes and short middle phalanges of the fifth fingers and the patient's OA and relatively small head might be separate phenomena.

Behavioural problems at school were the reason for re-evaluation at age 10 years. Attending a mainstream school (i.e. not one for children with special needs or disabilities), the boy did not have any obvious learning difficulties. Yet he displayed agitated and disruptive behaviour and was inclined to draw frightening things and to use a lot of fantasy in his play. He did not show agitated behaviour at home. His head circumference was now 50.7 centimetres (-1.5 SD).

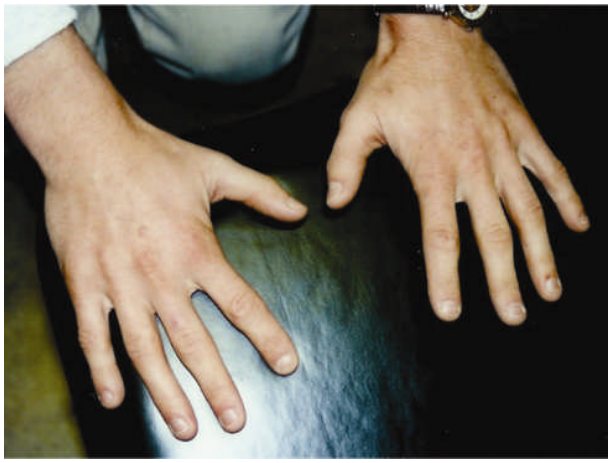
Figure 1 Clinical pictures (*for colour figure see page 227*)



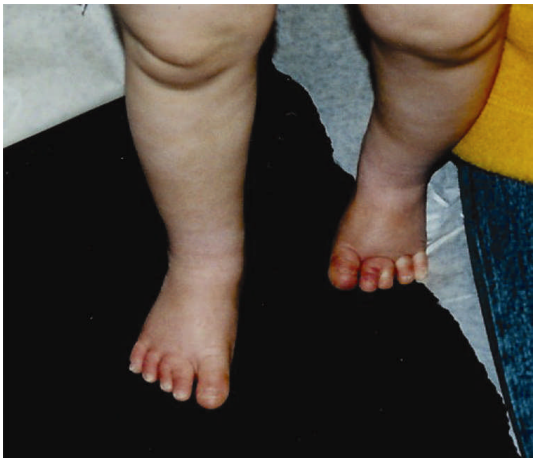
a *the proband's face*



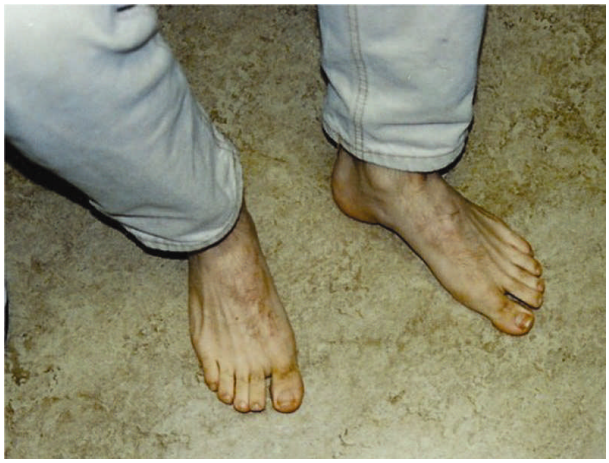
b *the proband's hands*



d *the father's hands*

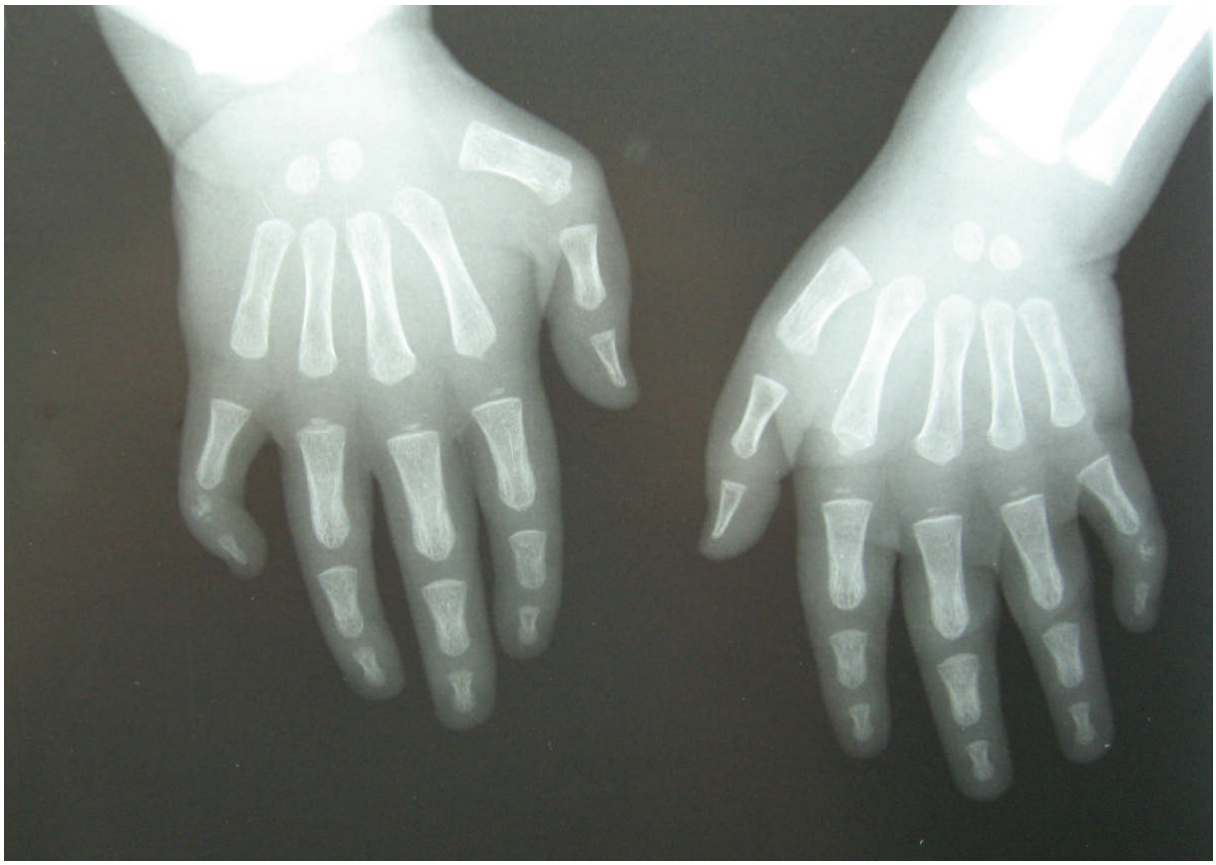


c *the proband's feet*



e *the father's feet.*

Figure 2 X-ray image of the hands of the proband, showing short middle phalanges of both fifth fingers with clinodactyly. See text for more details.

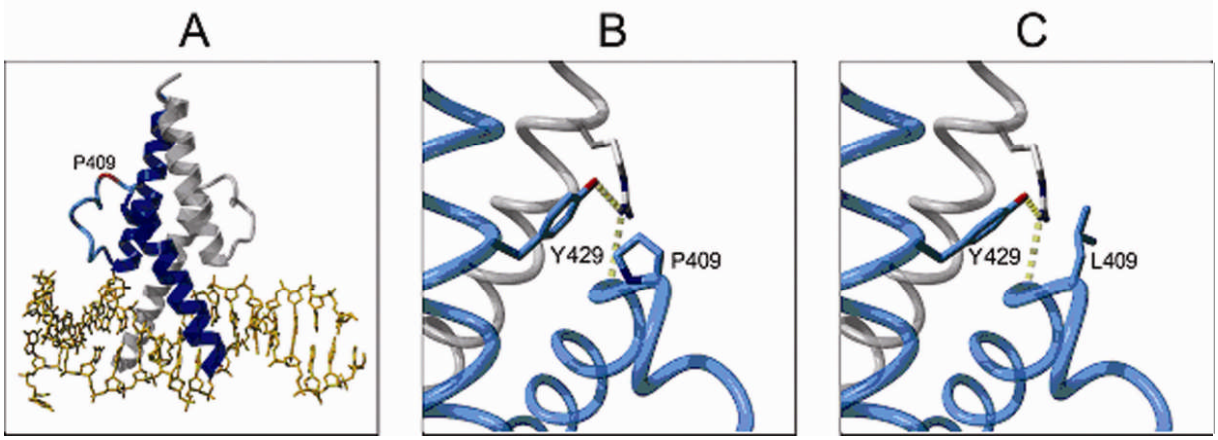


Since mutations in the *MYCN* gene had been reported to cause Feingold syndrome, sequence analysis was performed in the patient as described previously.[1] A heterozygous missense mutation c.1226C>T (p.Pro409Leu) in exon 3 of the *MYCN* gene was identified. This mutation was not present in the parents. Paternity was confirmed by haplotype analysis. Hence, this mutation has occurred *de novo*. The c.1226C>T mutation was not found in 100 ethnically-matched control individuals nor was it observed as polymorphism in SNP and EST databases. Furthermore, P409 is conserved from human to zebrafish,[14] (http://genome.ucsc.edu) and according to the SIFT[15] and PolyPhen[16] programs, the P409L substitution is predicted to be pathogenic.

The *MYCN* gene consists of three exons. The most important domains, among which the basic helix-loop-helix domain, are encoded by exon 3.[1] The proline at position 409 is located in the loop of the basic helix-loop-helix (bHLH) motif of MYCN as shown by computer-assisted molecular modeling (Figure 3a). P409 makes close hydrophobic contacts with a neighboring tyrosine side chain Y429. In the template MYC-MAX complex, the corresponding MYC tyrosine side chain hydrogen bonds with an arginine side chain in MAX. Due to the high sequence identity between MYC and MYCN this hydrogen bond is expected to also occur in a MYCN-MAX complex (Figure 3b). The P409L mutation does not seem to disturb this hydrogen bonding pattern as the model shows that there is sufficient space to accommodate the leucine side chain. This suggests that the

substitution does not have a large impact on local structure (Figure 3c). However, in the normal situation the distinctive cyclic structure of a proline side chain locks its ϕ backbone dihedral angle at approximately -60°, giving proline an exceptional conformational rigidity compared with other amino acids. This indicates that P409 is imposing a tertiary structural constraint on the bHLH domain of MYCN. Mutation of P409 to another amino acid would allow for additional freedom in the ϕ backbone dihedral angle at this position, which is likely to result in a less stable tertiary structure and hence affects the ability of MYCN to interact with MAX.

Figure 3 Molecular model of MYCN (*for colour figure see page 228*)



***A** Interaction of the MYCN-MAX complex with DNA. The two MYCN alpha-helices are shown in dark blue, the loop in lighter blue. MAX is shown in gray and the DNA in orange. The location of P409 in the HLH loop is indicated in red. **B**,**C** Detailed view of the P409L amino acid residue substitution. MYCN is shown in blue. The HLH motif of MAX is shown in gray. Hydrogen bonds are indicated by yellow dotted lines.*

Materials and Methods

Molecular modeling

To analyze the P409L mutation a molecular model of the MYCN helix-loop-helix (HLH) domain was constructed. The crystal structure of MYC-MAX bound to DNA, solved at 1.8-Å resolution (PDB entry 1NKP[17]), was used as a template for the model. The sequence identity between MYC and MYCN is 67% for the HLH domain. An initial model was built using SCWRL.[18] Subsequently, molecular dynamics simulations were run in explicit solvent with the Yamber2 force field and the associated protocol[19] until WHAT IF[20] quality indicators converged.

Discussion

This paper describes a heterozygous *de novo* c.1226C>T missense mutation in exon 3 of the *MYCN* gene, which predicts the substitution of a proline at position 409 with a leucine, in a child with clinical characteristics of Feingold syndrome. Pathogenicity of the

p.P409L missense mutation is supported by the absence of the mutation in healthy controls, conservation of P409, and molecular modelling of the mutation. In addition, a mutation study on the HLH domain of Id1 demonstrated that mutation of the corresponding proline in this HLH domain to different amino acids resulted in a 4-fold decrease of Id1 activity.[21]

Including the case of our patient, 14 different mutations in *MYCN* have been reported in the literature, of which 4 were missense and 10 were nonsense mutations.[1,4] In addition, whole-gene deletions have been described.[1,2] These mutations are predicted to result in a loss-of-function of MYCN activity. Both molecular modelling and the mutation study on the HLH domain of Id1 indicate that the p.P409L missense mutation fits this hypothesis, although we cannot exclude that the P409L mutation may exert dominant negative effects as well that contribute to the phenotype.

The patient's family members came to our attention only because the patient clearly showed severe congenital anomalies. During the diagnostic process, Feingold syndrome was suspected in the patient on clinical grounds. A detailed family history and physical examinations revealed minor characteristics possibly compatible with Feingold syndrome in several family members, including the patient's father.

There are two ways to interpret the limb anomalies. One, the oesophageal atresia and the relatively small head found in the patient should be considered symptoms of Feingold syndrome, whereas the limb anomalies are a familial anomaly present in the patient, his father and paternal aunt and grandmother. Alternatively, the patient’s limb anomalies may be part of his Feingold syndrome and be unassociated with the anomalies in his relatives. Mutational analysis in the parents excluded the possibility that the limb anomalies in the family members represented variable expression of Feingold syndrome.

The behavioural problems seen in the patient are difficult to interpret. Mild to moderate mental retardation or learning disabilities have been described in around 50% of patients with Feingold syndrome and microcephaly. Not much is known, however, about behavioural anomalies.[3,5,22-24] To the best of our knowledge, only Holder-Espinasse et al. described another patient with Feingold syndrome displaying behavioural difficulties, such as poor concentration and hyperactivity. Their report does not mention structural cranial abnormalities or the use of imaging techniques to exclude the presence of such abnormalities.[25] Our patient had no structural brain anomalies, as assessed by ultrasound at birth. No further imaging studies were done. As the spectrum of anomalies seen in Feingold syndrome is becoming more clear and patients with proven mutations in the *MYCN* gene are getting older, monitoring patients' behaviour and development could provide more insight into the behavioural phenotype of Feingold syndrome.[3,4,23]

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Part IV

Environmental Aspects

Environmental factors in the aetiology of oesophageal atresia and congenital diaphragmatic hernia: Results of a case-control study

Chapter 8

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ABSTRACT

Background
Oesophageal atresia with or without tracheo-oesophageal fistula (OA/TOF) and congenital diaphragmatic hernia (CDH) are severe congenital anomalies. Their aetiologies are mostly unknown and are thought to be multifactorial. No specific environmental factors have consistently been described as risk factors.

Methods
In a hospital-based study conducted during the years 2000 to 2004 in a paediatric surgical referral centre in the Netherlands, parents of children with OA/TOF or CDH and parents of a group of healthy children filled out a questionnaire about possible environmental risk factors in the month before or during the first trimester of pregnancy.

Results
Questionnaires were returned for 48 out of 69 cases (70%) with OA/TOF, for 75 out of 95 cases (79%) with CDH and for 202 out of 243 controls (83%). In OA/TOF, maternal age was significantly higher than in controls (32.2 vs. 30.6 years, p = 0.04). Gardening was borderline-significantly associated with OA/TOF in univariate analysis (OR = 2.0; 95% CI = 1.0 - 3.9) and remained borderline-significant in multivariate analysis (OR = 1.9; 95% CI = 1.0 - 3.7).
In univariate analysis, CDH was significantly associated with maternal use of alcohol (OR = 2.5; 95% CI = 1.4 - 4.3). Herbicide or insecticide use in the garden showed a borderline-significant association with CDH (OR = 2.2; 95% CI = 1.0 - 5.1). In logistic regression analysis, only maternal use of alcohol remained significant (OR = 2.4; 95% CI = 1.3 - 4.2).

Conclusions
We found a significant association between maternal alcohol use around the time of conception and CDH. A possible explanation might be the effect of alcohol on the retinoic acid pathway. A borderline-significant association was found between gardening and OA/TOF.

INTRODUCTION

Oesophageal atresia with or without tracheo-oesophageal fistula (OA/TOF) and congenital diaphragmatic hernia (CDH) are severe congenital anomalies that affect 1 in 3,500 and 1 in 3,000 newborns, respectively.[1,2] Not much is known about the causes of these congenital defects, apart from a few identifiable genetic abnormalities in selected cases.[3-7] Presently, each defect is thought to have a multifactorial aetiology, with both genetic and environmental factors playing a role.

Oesophageal atresia and/or tracheo-oesophageal fistula

Chromosomal anomalies have been reported in up to 10% of cases of OA/TOF, but no single specific chromosomal defect has been confirmed as an aetiological factor.[8] However, four genes, *MYCN*, *CHD7, MID1* and *SOX2,* have recently been implicated in rare cases of syndromic OA/TOF.[9-12]

Various environmental factors have been proposed as risk factors, such as exposure to exogenous sex hormones,[13,14] certain medications,[15,16] alcohol,[17] or infectious diseases.[18] Nevertheless, so far, involvement of any of these factors has not yet been confirmed.[19-24] When Adriamycin, an antibiotic used in chemotherapy, is given to pregnant rats during a specific developmental time window, considerable proportions of the offspring were found to have OA/TOF as part of a spectrum of defects in multiple organs in the offspring.[25] However, no such association has been reported for humans.[26,27]

Congenital diaphragmatic hernia

The evidence for a genetic contribution to the aetiology of CDH comes from several knockout mice models displaying diaphragm defects, and from reports of chromosomal abnormalities in small proportions of human CDH patients. The most important genes identified in animal models are *COUP-TFII*, *FOG2* and *GATA4*, which all reside in a chromosomal region deleted in a few human patients.[5,28,29] Mutations in *WT1*, *FOG2* and *STRA6* have been identified in humans.[5-7] Whether these genetic defects are sole causes of CDH, or merely provide a genetic background that interacts with environmental factors, remains to be established.

The first description of a possible environmental contribution to the aetiology of CDH was made in the early 1940s, when Andersen observed that vitamin A deficiency in pregnant rats causes CDH in the majority of their offspring.[30] Other environmental factors, such as zinc deficiency, have been studied as well, but none of these studies has provided solid evidence that any of these factors can cause CDH in humans.[31] The best-studied teratogen is the herbicide Nitrofen, which induces CDH in rats and mice when administered during the critical time-window of formation of the diaphragm and lungs in the developing embryo.[32,33] Nitrofen was shown to inhibit RALDH2, a key enzyme in the vitamin A pathway, providing more evidence for a role of this pathway in normal lung- and diaphragm development.[34]

An earlier paper from our group, which compared cases of OA/TOF to cases of CDH, found no specific environmental factors to be associated with either of these anomalies.[35] We cannot exclude the possibility, however, that other environmental factors not evaluated previously may predispose to the development of either OA/TOF or CDH. Therefore, the aim of this study was to generate hypotheses about possible environmental risk factors for OA/TOF and/or CDH based on data from questionnaires administered to the parents of cases and controls.

METHODS

Subjects

The Erasmus MC - Sophia Children's Hospital is a tertiary children's hospital and the paediatric surgery referral centre for the south-western Netherlands, which has a population of 4 million people and a birth rate of 35,000 infants per year. As one of the two ECMO centres in the country, it is also a national referral hospital for cases of CDH.
All singletons and twins treated in our hospital for OA/TOF or CDH and born during the five-year period from 2000 through 2004 were eligible for this study. The children's medical records were reviewed to retrieve general clinical data, anomaly-specific information and pedigree data.

We created a control group of children without birth defects, born during the same time period, who underwent minor surgery in our hospital (mostly repair of inguinal hernias, adeno-tonsillectomies or myringotomies). Controls were randomly chosen within the same age and gender category as the cases. We aimed to include 2 - 3 times as many controls as cases.

The study protocol was approved by the Erasmus MC Medical Ethics Review Board, and written informed consent was obtained from the parents for all cases and controls.

Questionnaires

As part of an ongoing study into the aetiology of OA/TOF and CDH, parents of children with these anomalies and parents of controls were asked to fill out a structured questionnaire consisting of 95 questions. As our aim was to perform an explorative study to generate hypotheses about possible risk factors, the questionnaire covered many different aspects of life, including medical and family history, demographic characteristics, pregnancy history, use of medication, diet, occupation and exposure to possible toxic substances, such as pesticides and insect-repellents, smoking, drugs and alcohol. All questions referred to the period from one month before conception to the end of the third month of pregnancy.

The data for this study were collected retrospectively. After informed consent was obtained, parents filled out the questionnaire. This questionnaire was a modified version

of a validated questionnaire used by the Californian Birth Defects Registry, translated and adapted to the Dutch situation.[36] From 2001 to 2004, parents of cases completed the questionnaire during the child's first admission. Parents of children born in 2000 received the questionnaire at home. The parents of controls received the questionnaire on the morning of admission of their child and filled it out the same day. Parents of controls who were unable to read or speak Dutch, or to understand English or French, were excluded from the study. If parents of cases had problems with the Dutch language, but could understand English or French, one of the researchers presented the questionnaire in the form of an interview performed in either language. The latter approach served to minimize the number of exclusions in the case-group.

This paper focuses on use of vitamin supplements (special prenatal vitamins, multivitamins, vitamin A and folate), exposure to paint, gardening activities, exposure to herbicides, insecticides or disinfectants, paternal or maternal smoking (active or passive) and maternal use of alcohol. These variables were defined as follows. Use of vitamin supplements: using the specified supplement at some stage during the month before or the first three months of pregnancy. Painting, gardening, exposure to herbicides, insecticides or disinfectants: experienced in the above specified period. Paternal or maternal smoking: any smoking during that period. Maternal passive smoking: maternal contact with people smoking in the house, at work or in public places. Maternal use of alcohol: any use of alcohol in the specified period. If alcohol use was reported, further information was gathered regarding its frequency.

All variables addressed in the questionnaire can be found in the appendix.

Statistical analysis

Analyses of cases with OA/TOF and of cases with CDH were carried out separately. We tested for differences in child characteristics between those cases for whom the parents responded and those for whom they did not.

Univariate analysis was performed using chi-square and Mann-Whitney tests where appropriate. Subsequently, some questions concerning similar exposures were combined. This was the case for: "vegetables, fruits or flowers grown within 1 km of the house", "herbicide or insecticide use in own garden" and "insecticide use anywhere", which were combined to form the variable "exposure to herbicides or insecticides". In addition, these variables were combined with the variable "disinfectant use in the house" to form the new variable "exposure to herbicides, insecticides or disinfectants". Results are given as odds ratios (OR) with 95% Confidence Intervals (95% CI).

If a significant difference between either OA/TOF or CDH and controls was found, the OA/TOF and CDH groups were also mutually compared to help estimate the possible effect of recall bias.

Variables that showed a significant or borderline-significant effect in univariate analysis were entered in multiple logistic regression analysis. P-values of < 0.05 were considered significant. Data were analysed using SPSS statistical software package version 12.0.1.

RESULTS

From January 2000 until December 2004, 69 children with OA/TOF and 95 children with CDH were admitted to our hospital. Questionnaires were completed for 70% (48/69) of cases with OA/TOF and for 79% (75/95) of cases with CDH. The response rate for the 243 controls was 83%. Reasons for non-participation included parents' insufficient command of the Dutch language and lack of time. All questionnaires but one were self-administered; in one case only it was necessary to resort to an interview.

In the OA/TOF group, birth weight, gestational age and number of twins did not significantly differ between responders and non-responders. The non-responding group, however, included significantly more parents of children who died (33% vs. 10%, p = 0.04), and significantly more parents of children with associated anomalies (100% vs. 73%, p = 0.01) (data not shown).

Table 1 shows the demographic characteristics of cases with OA/TOF compared to controls. The median age of the children with OA/TOF at the time the questionnaire was filled out was significantly lower than that of controls. Mothers of cases with OA/TOF were significantly older at the time of birth of their infant than mothers of controls.

In the CDH group, there were no significant differences between responders and non-responders in infant birth weight, gestational age, percentage of children who died, number of twins or number of children with associated anomalies (data not shown).

Table 2 shows the demographic characteristics of cases with CDH compared to controls. There was a significant difference between cases with CDH and controls in the age of the child at the time of filling out the questionnaire, with cases being younger than controls. Maternal age at birth tended to be higher for the children with CDH as compared to controls, but the difference failed to reach statistical significance.

For lack of data the testing procedure for differences between responders and non-responders could not be performed for the controls.

Tables 3 and 4 show the results of the univariate analysis for cases with OA/TOF compared to controls and for cases with CDH compared to controls, respectively.

Table 1 Distribution of demographic characteristics of 48 cases with OA/TOF and 202 unaffected controls

Variable	OA/TOF	Controls	p-value
Year of birth			0.87
2000	8 (17)	31 (15)	
2001	10 (21)	44 (22)	
2002	13 (27)	42 (21)	
2003	7 (15)	32 (16)	
2004	10 (21)	53 (26)	
M : F ratio	1.4 : 1	1.3 : 1	0.99
Child's age at questionnaire (days)	189 (3 - 1487)	522 (23 - 2056)	< 0.001
Maternal age at birth of child (years)	32.2 (4.5)	30.6 (5.1)	0.04
Maternal BMI before pregnancy (kg/cm^2)	21.9 (16.4 - 41.5)	22.9 (14.1 - 40.0)	0.23
Paternal age at birth of child (years)	35 (24 - 51)	33 (17 - 71)	0.10
Gravidity	2 (1 - 5)	2 (1 - 6)	0.20
Birth weight (grams)	2880 (1280 - 4000)	3200 (590 - 5170)	0.10
Gestational age (weeks)	38.5 (29.6 - 42.4)	39.0 (24.9 - 42.1)	0.81
Mother employed	38 (79)	147 (73)	0.47
Father employed	40 (87)	175 (89)	0.85

M: male; F: female; BMI: body mass index; kg: kilograms; cm: centimetres
Missing values were excluded. Numbers of missing values (OA/TOF; controls) were:
Child's age at questionnaire: 6; 0. Maternal age at birth of child: 0; 2. Maternal BMI before pregnancy: 4; 13. Paternal age at birth of child: 1; 8. Gravidity: 1; 39. Birth weight: 1; 19. Gestational age: 0; 13. Mother employed: 0;0. Father employed: 2; 6.
Year of birth: numbers represent total number of children in that group (%)
Child's age at questionnaire, maternal BMI, paternal age, gravidity, birth weight, gestational age: represented as median (minimum – maximum)
Maternal age: represented as mean (standard deviation)
Mother (Father) employed: numbers represent number of children (%) whose mother (father) had a job in the month before or the first three months of pregnancy.

Oesophageal atresia / tracheo-oesophageal fistula

Gardening during the first trimester and/or the month before conception showed a borderline-significant difference between cases and controls (OR = 2.0; 95% CI = 1.0 - 3.9, Table 3). There was no significant difference between cases with CDH and cases with OA/TOF for this variable (data not shown). Gardening and maternal age were entered into the multivariate analysis. Gardening remained of borderline-significance (OR = 1.9; 95% CI = 1.0 - 3.7). Maternal age lost its significance, but remained of borderline-significance (OR = 1.1; 95% CI = 1.0 - 1.2).

Congenital diaphragmatic hernia

Of the possible aetiological variables studied, herbicide or insecticide use in the garden showed a borderline-significant association with CDH (Table 4). No significant association was found for this variable between cases with CDH and cases with OA/TOF (data not shown).

Table 2 Distribution of demographic characteristics of 75 cases with CDH and 202 unaffected controls

Variable	CDH	Controls	p-value
Year of birth			0.29
2000	16 (21)	31 (15)	
2001	14 (19)	44 (22)	
2002	12 (16)	42 (21)	
2003	18 (24)	32 (16)	
2004	15 (20)	53 (26)	
M : F	1.1 : 1	1.3 : 1	0.69
Child's age at questionnaire (days)	86 (0 - 1293)	522 (23 - 2056)	< 0.001
Maternal age at birth of child (years)	31.8 (5.0)	30.6 (5.1)	0.08
Maternal BMI before pregnancy (kg/cm^2)	22.7 (18.1 - 38.1)	22.9 (14.1 - 40.0)	0.58
Paternal age at birth of child (years)	34 (20 - 52)	33 (17 - 71)	0.18
Gravidity	2 (1 - 7)	2 (1 - 6)	0.11
Birth weight (grams)	2968 (1300 - 4000)	3200 (590 - 5170)	0.16
Gestational age (weeks)	38.6 (29.7 - 42.0)	39.0 (24.9 - 42.1)	0.97
Mother employed	55 (73)	147 (73)	1.00
Father employed	63 (84)	175 (89)	0.33

M: male; F: female; BMI: body mass index; kg: kilograms; cm: centimetres
Missing values were excluded. Numbers of missing values (CDH; controls) were:
Child's age at questionnaire: 5; 0. Maternal age at birth of child: 1; 2. Maternal BMI before pregnancy: 4; 13. Paternal age at birth of child: 0; 8. Gravidity: 2; 39. Birth weight: 7; 19. Gestational age: 4; 13. Mother employed: 0;0. Father employed: 0; 6.
Year of birth: numbers represent total number of children in that group (%).
Child's age at questionnaire, maternal BMI, paternal age, gravidity, birth weight, gestational age: represented as median (minimum – maximum)
Maternal age: represented as mean (standard deviation)
Mother (Father) employed: numbers represent number of children (%) whose mother (father) had a job in the month before or the first three months of pregnancy.

A highly significant association was found for maternal use of any alcohol during the first trimester and/or the month before conception and CDH, with an OR of 2.5 (95% CI: 1.4 - 4.3). There were no significant differences between cases with CDH and cases with OA/TOF (data not shown). Analyses taking into account frequency of alcohol use during the susceptible period of pregnancy showed a slightly higher risk for those mothers with the most frequent consumption (OR = 3.0; 95% CI = 1.1 - 8.0), but confidence intervals overlapped (Table 5). Adjustment for maternal age did not affect the significance.

The following variables were included in the logistic regression model: maternal age, use of herbicides or insecticides in the garden, and maternal use of alcohol. The results are shown in Table 6. While maternal age and use of herbicides or insecticides were of borderline significance, only maternal use of alcohol showed a strong significant association with CDH (OR = 2.4; 95% CI = 1.3 - 4.2).

DISCUSSION

This paper describes the results of a study of environmental factors as risk factors in the aetiology of OA/TOF and CDH.

Oesophageal atresia/tracheo-oesophageal fistula

Maternal age was significantly higher for cases than for controls. In epidemiological studies, higher maternal age is sometimes, but not consistently, found to be more frequent in OA/TOF. This is in part explained, however, by the increased risk of having a child with an aneuploidy, such as trisomy 21, with advanced maternal age; trisomic children have a higher chance of developing several congenital anomalies, including OA/TOF.[1,8] Maternal age in the present study remained borderline significant in multivariate analysis.

Gardening showed a trend towards significance. No significant differences were found between the CDH group and controls or between the CDH and OA/TOF groups, arguing against recall bias. In 1988, McDonald et al. reported a higher risk for OA/TOF among children of mothers who worked in agriculture or horticulture and hypothesized that this might have been due to exposure to pesticides; however, because of the small number of exposed cases (n = 2), no conclusion could be drawn.[37] In our study, herbicides and insecticides did not show a significant association with the occurrence of OA/TOF.

Congenital diaphragmatic hernia

Herbicide or insecticide use in the garden showed a borderline-significant difference between cases with CDH and controls, but no difference between CDH cases and OA/TOF cases or between OA/TOF cases and controls, arguing against recall bias. In the logistic regression analysis, this factor stayed of borderline-significance. The only variable that consistently showed a significant difference between cases and controls, both in univariate and in multivariate analysis, was maternal alcohol use. Again, analysis of cases with OA/TOF versus controls showed no significant difference, making recall bias unlikely. As shown in Table 5, no significant dose-response relationship could be found in our population. Our data suggest that drinking any alcohol, independent of how frequently or how much, seems to have an association with CDH in the foetus.

To the best of our knowledge, maternal alcohol consumption has not been described as a risk factor for CDH in the literature. However, from a biological point of view, this relationship would not be implausible. Vitamin A metabolism is an important regulator of many developmental processes and is thought to be very important in diaphragm and lung development. Disturbances of the vitamin A pathway have been associated with diaphragmatic defects in animal models (e.g. in the vitamin A deficient and the Nitrofen rat models) and mutations in the *STRA6* gene, which is involved in the vitamin A pathway, have been described in humans with CDH.[6,30,32,34,38]

Table 3 Prevalence (%) and univariate analysis of exposures studied in 48 cases with OA/TOF and 202 controls

Variable	Cases with OA/TOF (n = 48)		Controls (n = 202)		OR (95% CI)*
	n	%	n	%	
Vitamin supplements					
Prenatal vitamins					
No	26	70	113	74	Reference
Yes	11	30	40	26	1.2 (0.5 - 2.6)
Multivitamins					
No	27	66	113	74	Reference
Yes	14	34	39	26	1.5 (0.7 - 3.2)
Vitamin A					
No	35	97	135	96	Reference
Yes	1	3	5	4	0.8 (0.1 - 6.8)
Folate					
No	13	29	45	24	Reference
Yes	32	71	140	76	0.8 (0.4 - 1.6)
Exposures					
Painting					
No	28	72	140	82	Reference
Yes	11	28	30	18	1.8 (0.8 - 4.1)
Gardening					
No	23	52	121	69	Reference
Yes	21	48	55	31	2.0 (1.0 - 3.9)
Vegetables, fruits or flowers grown within 1 km of the house					
No	28	67	140	79	Reference
Yes	14	33	37	21	1.9 (0.9 - 4.0)
Herbicide or insecticide use in own garden					
No	37	86	174	92	Reference
Yes	6	14	15	8	1.9 (0.7 - 5.2)

Insecticide use anywhere					
No	43	96	178	91	Reference
Yes	2	4	17	9	0.5 (0.1 - 2.2)
Disinfectant use in house					
No	25	53	105	57	Reference
Yes	22	47	79	43	1.2 (0.6 - 2.2)
Exposures, combined variables (see text for details)					
Exposure to herbicides or insecticides					
No	20	51	115	67	Reference
Yes	19	49	57	33	1.9 (0.9 - 3.9)
Exposure to herbicides, insecticides or disinfectants					
No	12	27	58	35	Reference
Yes	33	73	110	65	1.5 (0.7 - 3.0)
Life style factors					
Maternal alcohol					
No	24	50	122	62	Reference
Yes	24	50	74	38	1.6 (0.9 - 3.1)
Paternal smoking					
No	26	57	119	62	Reference
Yes	20	43	74	38	1.2 (0.6 - 2.4)
Maternal smoking					
No	38	83	157	82	Reference
Yes	8	17	35	18	0.9 (0.4 - 2.2)
Maternal passive smoking					
No	12	27	65	35	Reference
Yes	33	73	120	65	1.5 (0.7 - 3.1)

All variables refer to the period from 1 month before pregnancy to the end of the first trimester.
Numbers do not always add up to total numbers due to missing data.
** ORs remained similar after adjustment for maternal age, except for gardening, which lost its significance, but remained borderline significant (see text for details)*
km: kilometer

Table 4 Prevalence (%) and univariate analysis of exposures studied in 75 cases with CDH and 202 controls

Variable	Cases with CDH (n = 75)		Controls (n = 202)		OR (95% CI)*
	n	%	n	%	
Vitamin supplements					
Prenatal vitamins					
No	41	68	113	74	Reference
Yes	19	32	40	26	1.3 (0.7 - 2.5)
Multivitamins					
No	40	63	113	74	Reference
Yes	23	37	39	26	1.7 (0.9 - 3.1)
Vitamin A					
No	55	98	135	96	Reference
Yes	1	2	5	4	0.5 (0.1 - 4.3)
Folate					
No	14	19	45	24	Reference
Yes	58	81	140	76	1.3 (0.7 - 2.6)
Exposures					
Painting					
No	51	78	140	82	Reference
Yes	14	22	30	18	1.3 (0.6 - 2.6)
Gardening					
No	41	59	121	69	Reference
Yes	29	41	55	31	1.6 (0.9 - 2.8)
Vegetables, fruits or flowers grown within 1 km of the house					
No	50	76	140	79	Reference
Yes	16	24	37	21	1.2 (0.6 - 2.4)
Herbicide or insecticide use in own garden					
No	58	84	174	92	Reference
Yes	11	16	15	8	2.2 (1.0 - 5.1)
Insecticide use anywhere					

No	63	89	178	91	Reference
Yes	8	11	17	9	1.3 (0.5 - 3.2)
Disinfectant use in house					
No	38	55	105	57	Reference
Yes	31	45	79	43	1.1 (0.6 - 1.9)
Exposures, combined variables (see text for details)					
Exposure to herbicides or insecticides					
No	37	59	115	67	Reference
Yes	26	41	57	33	1.4 (0.8 - 2.6)
Exposure to herbicides, insecticides or disinfectants					
No	24	36	58	35	Reference
Yes	42	64	110	65	0.9 (0.5 - 1.7)
Life style factors					
Maternal alcohol					
No	30	40	122	62	Reference
Yes	45	60	74	38	2.5 (1.4 - 4.3)
Paternal smoking					
No	50	67	119	62	Reference
Yes	25	33	74	38	0.8 (0.5 - 1.4)
Maternal smoking					
No	63	88	157	82	Reference
Yes	9	13	35	18	0.6 (0.3 - 1.4)
Maternal passive smoking					
No	19	28	65	35	Reference
Yes	49	72	120	65	1.4 (0.8 - 2.6)

All variables refer to the period from 1 month before pregnancy to the end of the first trimester.
Numbers do not always add up to total numbers due to missing data.
** ORs remained similar after adjustment for maternal age*
km: kilometer

Table 5 Distribution (%) of frequency of alcohol use among mothers of 75 cases with CDH and 202 controls

Frequency	CDH (n = 75)		Controls (n = 202)		OR (95% CI)
	n	%	n	%	
Never	30	40	122	62	Reference
1-3 times in the entire period	30	40	50	26	2.4 (1.3 - 4.5)
1-3 times per month	7	9	13	7	2.2 (0.8 - 6.0)
Multiple times per week	8	11	11	6	3.0 (1.1 - 8.0)

Missing values in controls: n = 6.

Table 6 Multiple logistic regression analysis for 75 cases with CDH and 202 controls (see text for details)

Variable	OR (95% CI)
Maternal age at birth	1.0 (1.0 - 1.1)[a]
Herbicide or insecticide use in garden	2.2 (0.9 - 5.2)[b]
Maternal alcohol	2.3 (1.3 - 4.2)[b]

a: per year
b: yes versus no

The vitamin A pathway shares characteristics with the alcohol pathway. Retinol, the main dietary source of vitamin A in humans, is oxidized to retinal by alcohol dehydrogenases (ADHs), in particular by ADH1. Retinal is then oxidized to retinoic acid by aldehyde dehydrogenases (ALDHs), also known as retinaldehyde dehydrogenases (RALDHs). Retinoic acid is the substrate for the retinoic acid receptors RAR and RXR, which mediate the effects of vitamin A in the cell.
In a similar manner, ethanol is oxidized to acetaldehyde and further to acetic acid by ADHs and ALDHs, respectively. In the presence of ethanol, retinol metabolism is inhibited due to substrate competition. In addition, ethanol induces enzymes that increase degradation of retinoic acid.[39-41]

Thus, we hypothesize that the use of alcohol at a vulnerable time in pregnancy could lead through substrate competition with retinol and increased degradation of retinoic acid to a relative vitamin A deficiency causing diaphragmatic defects. The individual genetic backgrounds of the foetus and the mother may determine the severity of the effect, if any. Interestingly, vitamin A supplementation during pregnancy was non-significantly inversely associated with the development of CDH (see Tables 3 and 4), but numbers in all groups were very small. Also, we did not have sufficient information to determine the exact calendar time during pregnancy at which the mother took these supplements.

However, CDH is not usually described as a feature of the fetal alcohol spectrum disorder.[42-44] In addition, we did not find a dose-response relationship, which might have been expected considering that alcohol and retinol compete for binding sites. These two facts would argue against the association found in our study being real.

Strengths and weaknesses of this study

Case ascertainment in this study was very complete. No cases admitted to the hospital were missed, all parents were asked to participate and diagnosis was always certain, as verified by complete medical record review.

As a consequence of its hypothesis-generating nature, the questionnaire used in this study covered a wide range of exposures, enabling us to explore multiple potential aetiological factors. However, this characteristic also introduces the possibility of finding associations resulting from multiple testing. Therefore, our findings should be tested by repeating the study in a different population for confirmation.

All exposures studied were self-reported by the mothers. For vitamins/supplements, only their use was recorded, but no information was gathered about the dosage, frequency or duration of supplementation.

As mothers were asked to recall events that occurred some time back (see Tables 1 and 2), responses may have lacked precision. Mothers of affected children may have been more likely to recall adverse events during pregnancy, which may have introduced a recall bias. In addition, the time elapsed between the birth of the child and completing the questionnaire was significantly shorter for cases with OA/TOF or CDH than for controls. Case mothers therefore may have had a more complete recollection of the events during pregnancy.
However, there is no reason to believe that case mothers had preconceived ideas about associations between specific exposures and the anomalies found in their child. As the questionnaire was very extensive and covered a multitude of aspects of maternal and family life, a bias towards reporting a specific exposure seems less likely. Yet, although recall bias has been shown not to play an important role in case-control studies looking at pregnancy outcomes and exposures, it can never be completely excluded.[45,46] We have also tried to gain insight into a possible effect of recall bias by comparing two groups of malformed infants mutually and to non-malformed controls. As both have severe congenital anomalies, the same recall bias would be expected from cases with OA/TOF as from cases with CDH.

There were no differences in child characteristics between responders and non-responders in the CDH group. For OA/TOF, however, the non-responding group included significantly more mothers of children who died and children who had associated anomalies. Thus, as the more severely affected patients seem to have been underrepresented in the OA/TOF case group, this may have led to a bias.

In general, searching for causes of congenital anomalies is fundamentally intricate. Both OA/TOF and CDH may present in any of three forms: as isolated anomalies, as part of a complex of congenital defects, or within the context of a known syndrome or association. Genetic, environmental and maternal metabolic factors may play a role in all three forms - in varying degrees of importance. A wide range of environmental factors has been described in the aetiology of congenital anomalies in general.

Orford et al. have suggested that it is not so much the nature but rather the timing of the adverse event during pregnancy that causes the anomaly. Different events occurring at one specific time then could result in the same anomaly.[47]

Gene-environment interactions have become a focus of research in recent years.[48-50] Individual (genetic) differences in rates of metabolism of toxic substances may well determine the amount of potentially damaging substances that reach the foetus. This would then explain why one specific exposure should not have the same effect in all foetuses.

The significant association between maternal alcohol intake and CDH found in this study fully supports the conclusions of a recently published report of the Health Council of the Netherlands, in which alcohol consumption in pregnancy is completely discouraged.[51] We hesitate, however, to draw definite conclusions from our study, not only because CDH is not normally found in the fetal alcohol syndrome, but also because we could not demonstrate a dose-response relationship for alcohol consumption.

It follows that more studies are needed to gain additional insight in these processes and to highlight specific environmental factors in the aetiology of these severe congenital anomalies.

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Appendix - table 1 Prevalence (%) and univariate analysis of exposures studied in 48 cases with OA/TOF and 202 controls (all factors included)

Variable	Cases with OA/TOF (n = 48)		Controls (n = 202)		OR (95% CI)*
	n	%	n	%	
Illnesses					
Maternal diabetes					
No	46	98	191	96	Reference
Yes	1	2	8	4	0.5 (0.1 - 4.3)
Maternal epilepsy					
No	47	98	188	98	Reference
Yes	1	2	3	2	1.3 (0.1 - 13.1)
Maternal fever					
No	43	93	176	96	Reference
Yes	3	7	8	4	1.5 (0.4 - 6.0)
Vaginal discharge or cystitis					
No	39	87	156	78	Reference
Yes	6	13	43	22	0.6 (0.2 - 1.4)
GP consultation					
No	31	69	146	77	Reference
Yes	14	31	44	23	1.5 (0.7 - 3.1)
Medication					
Valium					
No	47	100	191	97	N/a
Yes	0	0	6	3	N/a
Valproic acid					
No	43	100	184	99	N/a
Yes	0	0	1	1	N/a
Contraceptive pill					
No	44	96	183	92	Reference
Yes	2	4	16	8	0.5 (0.1 - 2.3)
Vitamin supplements					
Prenatal vitamins					
No	26	70	113	74	Reference
Yes	11	30	40	26	1.2 (0.5 - 2.6)
Multivitamins					
No	27	66	113	74	Reference
Yes	14	34	39	26	1.5 (0.7 - 3.2)

Vitamine A					
No	35	97	135	96	Reference
Yes	1	3	5	4	0.8 (0.1 - 6.8)
Folate					
No	13	29	45	24	Reference
Yes	32	71	140	76	0.8 (0.4 - 1.6)
Other					
No	31	94	99	94	Reference
Yes	2	6	6	6	1.1 (0.2 - 5.5)
Diet					
Before pregnancy					
No	45	96	198	98	Reference
Yes	2	4	4	2	2.2 (0.4 - 12.4)
During pregnancy					
No	45	96	197	98	Reference
Yes	2	4	4	2	2.2 (0.4 - 12.3)
Exposures					
Electric blanket or bed heater					
No	41	91	177	89	Reference
Yes	4	9	21	11	0.8 (0.3 - 2.5)
Activities involving possible toxic exposure					
Repairing cars					
No	39	100	158	99	N/a
Yes	0	0	2	1	N/a
Pottery					
No	39	100	159	99	N/a
Yes	0	0	1	1	N/a
Developing photographs					
No	38	95	159	98	Reference
Yes	2	5	3	2	2.8 (0.5 - 17.3)
Sculpturing					
No	39	100	158	99	N/a
Yes	0	0	1	1	N/a
Metal work					
No	39	100	158	99	N/a
Yes	0	0	2	1	N/a
Jewellery making					
No	38	97	157	98	Reference
Yes	1	3	3	2	1.4 (0.1 - 13.6)

	n	%	n	%	
Burning off paint					
No	38	100	159	99	N/a
Yes	0	0	2	1	N/a
Leather working					
No	39	100	160	100	N/a
Yes	0	0	0	0	N/a
Painting					
No	28	72	140	82	Reference
Yes	11	28	30	18	1.8 (0.8 - 4.1)
Gardening					
No	23	52	121	69	Reference
Yes	21	48	55	31	2.0 (1.0 - 3.9)
Vegetables, fruits or flowers grown within 1 km of the house					
No	28	67	140	79	Reference
Yes	14	33	37	21	1.9 (0.9 - 4.0)
Herbicide or insecticide use in own garden					
No	37	86	174	92	Reference
Yes	6	14	15	8	1.9 (0.7 - 5.2)
Insecticide use anywhere					
No	43	96	178	91	Reference
Yes	2	4	17	9	0.5 (0.1 - 2.2)
Disinfectant use in house					
No	25	53	105	57	Reference
Yes	22	47	79	43	1.2 (0.6 - 2.2)
Exposures, combined variables					
Exposure to herbicides or insecticides					
No	20	51	115	67	Reference
Yes	19	49	57	33	1.9 (0.9 - 3.9)
Exposure to herbicides, insecticides or disinfectants					
No	12	27	58	35	Reference
Yes	33	73	110	65	1.5 (0.7 - 3.0)
Life style factors					
Maternal smoking					
No	38	83	157	82	Reference
Yes	8	17	35	18	0.9 (0.4 - 2.2)
Passive smoking					
No	12	27	65	35	Reference
Yes	33	73	120	65	1.5 (0.7 - 3.1)

Maternal alcohol					
No	24	50	122	62	Reference
Yes	24	50	74	38	1.6 (0.9 - 3.1)
Maternal softdrugs or harddrugs					
No	48	100	191	98	N/a
Yes	0	0	3	2	N/a
*Exposure to possibly toxic substances at mother's work***					
No	24	69	102	75	Reference
Yes	11	31	34	25	1.4 (0.6 - 3.1)
*Use of computer at mother's work***					
No	8	22	31	22	Reference
Yes	28	78	110	78	1.0 (0.4 - 2.4)
*Exposure to possibly toxic substances at father's work***					
No	28	80	136	87	Reference
Yes	7	20	20	13	1.7 (0.7 - 4.4)
Paternal smoking					
No	26	57	119	62	Reference
Yes	20	43	74	38	1.2 (0.6 - 2.4)
Family history					
Structural birth defects in 1st degree family members					
No	38	90	150	90	Reference
Yes	4	10	17	10	0.9 (0.3 - 2.9)
Structural birth defects in 2nd degree family members					
No	33	94	137	94	Reference
Yes	2	6	8	6	1.0 (0.2 - 5.1)
2 or more previous miscarriages					
No	43	96	187	97	Reference
Yes	2	4	5	3	1.7 (0.3 - 9.3)

All variables refer to the period of 1 month before pregnancy until the end of the first trimester, except maternal diabetes and maternal epilepsy, which were not restricted to a specific period, and family history
Numbers do not always add up to total numbers due to missing data.
** ORs remained similar after adjustment for maternal age, except for gardening, which lost its significance, but stayed borderline significant (see text for details)*
*** Only for working mothers/fathers*
GP: general practitioner; N/a: not applicable; km: kilometre

Appendix - table 2 Prevalence (%) and univariate analysis of exposures studied in 75 cases with CDH and 202 controls (all factors included)

Variable	Cases with CDH (n = 75)		Controls (n = 202)		OR (95% CI)*
	n	%	n	%	
Illnesses					
Maternal diabetes					Reference
No	66	90	191	96	2.5 (0.9 – 7.3)
Yes	7	10	8	4	
Maternal epilepsy					Reference
No	72	99	188	98	0.9 (0.1 – 8.5)
Yes	1	1	3	2	
Maternal fever					Reference
No	62	90	176	96	2.5 (0.9 – 7.1)
Yes	7	10	8	4	
Vaginal discharge or cystitis					Reference
No	59	81	156	78	0.9 (0.4 – 1.7)
Yes	14	19	43	22	
GP consultation					Reference
No	48	70	146	77	1.5 (0.8 – 2.7)
Yes	21	30	44	23	
Medication					
Valium					
No	73	100	191	97	N/a
Yes	0	0	6	3	N/a
Valproic acid					
No	68	100	184	99	N/a
Yes	0	0	1	1	N/a
Contraceptive pill					
No	72	96	183	92	Reference
Yes	3	4	16	8	0.5 (0.1 – 1.7)
Vitamin supplements					
Prenatal vitamins					
No	41	68	113	74	Reference
Yes	19	32	40	26	1.3 (0.7 – 2.5)
Multivitamins					
No	40	63	113	74	Reference
Yes	23	37	39	26	1.7 (0.9 – 3.1)

Vitamine A					
No	55	98	135	96	Reference
Yes	1	2	5	4	0.5 (0.1 – 4.3)
Folate					
No	14	19	45	24	Reference
Yes	58	81	140	76	1.3 (0.7 – 2.6)
Other					
No	47	92	99	94	Reference
Yes	4	8	6	6	1.4 (0.4 – 5.2)
Diet					
Before pregnancy					
No	68	92	198	98	Reference
Yes	6	8	4	2	4.4 (1.2 – 15.9)
During pregnancy					
No	65	87	197	98	Reference
Yes	10	13	4	2	7.6 (2.3 – 25.0)
Exposures					
Electric blanket or bed heater					
No	62	85	177	89	Reference
Yes	11	15	21	11	1.5 (0.7 – 3.3)
Activities involving possible toxic exposure					
Repairing cars					
No	64	100	158	99	N/a
Yes	0	0	2	1	N/a
Pottery					
No	62	97	159	99	Reference
Yes	2	3	1	1	5.1 (0.5 – 57.6)
Developing photographs					
No	60	94	159	98	Reference
Yes	4	6	3	2	3.5 (0.8 – 16.3)
Sculpturing					
No	64	100	158	99	N/a
Yes	0	0	1	1	N/a
Metal work					
No	64	100	158	99	N/a
Yes	0	0	2	1	N/a
Jewellery making					
No	64	100	157	98	N/a
Yes	0	0	3	2	N/a

	n	%	n	%	
Burning off paint					
No	63	97	159	99	Reference
Yes	2	3	2	1	2.5 (0.3 – 18.3)
Leather working					
No	63	98	160	100.0	N/a
Yes	1	2	0	0.0	N/a
Painting					
No	51	78	140	82	Reference
Yes	14	22	30	18	1.3 (0.6 – 2.6)
Gardening					
No	41	59	121	69	Reference
Yes	29	41	55	31	1.6 (0.9 – 2.8)
Vegetables, fruits or flowers grown within 1 km of the house					
No	50	76	140	79	Reference
Yes	16	24	37	21	1.2 (0.6 – 2.4)
Herbicide or insecticide use in own garden					
No	58	84	174	92	Reference
Yes	11	16	15	8	2.2 (1.0 – 5.1)
Insecticide use anywhere					
No	63	89	178	91	Reference
Yes	8	11	17	9	1.3 (0.5 – 3.2)
Disinfectant use in house					
No	38	55	105	57	Reference
Yes	31	45	79	43	1.1 (0.6 – 1.9)
Exposures, combined variables					
Exposure to herbicides or insecticides					
No	37	59	115	67	Reference
Yes	26	41	57	33	1.4 (0.8 – 2.6)
Exposure to herbicides, insecticides or disinfectants					
No	24	36	58	35	Reference
Yes	42	64	110	65	0.9 (0.5 – 1.7)
Life style factors					
Maternal smoking					
No	63	88	157	82	Reference
Yes	9	13	35	18	0.6 (0.3 – 1.4)
Passive smoking					
No	19	28	65	35	Reference
Yes	49	72	120	65	1.4 (0.8 – 2.6)

Maternal alcohol					
No	30	40	122	62	Reference
Yes	45	60	74	38	2.5 (1.4 – 4.3)
Maternal softdrugs or harddrugs					
No	75	100	191	98	N/a
Yes	0	0	3	2	N/a
*Exposure to possibly toxic substances at mother's work***					
No	42	81	102	75	Reference
Yes	10	19	34	25	0.7 (0.3 – 1.6)
*Use of computer at mother's work***					
No	18	35	31	22	Reference
Yes	33	65	110	78	0.5 (0.3 – 1.0)
*Exposure to possibly toxic substances at father's work***					
No	48	83	136	87	Reference
Yes	10	17	20	13	1.4 (0.6 – 3.2)
Paternal smoking					
No	50	67	119	62	Reference
Yes	25	33	74	38	0.8 (0.5 – 1.4)
Family history					
Structural birth defects in 1st degree family members					
No	63	91	150	90	Reference
Yes	6	9	17	10	0.8 (0.3 – 2.2)
Structural birth defects in 2nd degree family members					
No	56	89	137	94	Reference
Yes	7	11	8	6	2.1 (0.7 – 6.2)
2 or more previous miscarriages					
No	71	96	187	97	Reference
Yes	3	4	5	3	1.6 (0.4 – 6.8)

All variables refer to the period of 1 month before pregnancy until the end of the first trimester, except maternal diabetes and maternal epilepsy, which were not restricted to a specific period, and family history.
Numbers do not always add up to total numbers due to missing data.
** ORs remained similar after adjustment for maternal age*
*** Only for working mothers/fathers*
GP: general practitioner; N/a: not applicable; km: kilometre

Oesophageal atresia and tracheo-oesophageal fistula in children of women exposed to diethylstilbestrol *in utero*

Chapter 9

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ABSTRACT

Objective
To study the possible risk to mothers exposed *in utero* to diethylstilbestrol (DES) for offspring with oesophageal atresia/tracheo-oesophageal fistula (OA/TOF).

Study design
Information on mother's *in utero* exposure to DES was obtained from three sources: questionnaires completed by members of the parents' association of children with OA/TOF; records of patients with OA/TOF from a hospital database; files from the Northern Netherlands EUROCAT birth defects registry.

Results
3 of 124 (2.4%) mothers from the parents' association and 6 of 192 (3.1%) mothers from the hospital cases reported *in utero* exposure to DES. For 8848 children registered by EUROCAT, 33 (0.37%) mothers reported *in utero* exposure to DES. Of the 117 infants with OA/TOF, 4 (3.4%) had a mother with *in utero* exposure to DES; this association was statistically significant (P = 0.001).

Conclusion
We report a possible transgenerational effect of DES exposure in the aetiology of some cases of OA/TOF.

INTRODUCTION

Oesophageal atresia (OA) and associated tracheo-oesophageal fistula (TOF) are severe congenital anomalies affecting approximately 2.9 per 10,000 newborns.[1] Associated anomalies occur in about 50% of cases.[1] The best-known association of anomalies has the acronym VACTERL (Vertebral, Anal, Cardiac, Tracheo-oEsophageal, Renal, Limb). Little is known about the aetiology of OA/TOF. Chromosomal anomalies have been reported to be present in 10% of cases of OA/TOF.[1] A few genes have been implicated in the aetiology of syndromic OA/TOF, namely *MYCN*, *SOX2, CHD7* en *MID1* (for review see Brunner and Van Bokhoven, 2005).[2]

Knockout studies in mice have elucidated the functions of different genes involved in foregut development, implicating genes like *Sonic hedgehog*, *Gli2*, *Gli3*, *Foxf1* and *Ttf-1* in the development of OA/TOF (for review see Felix et al.).[3] However, other than one case report of a deletion of *SHH*, no mutations in these genes have been reported in humans with OA or TOF.[4]

Environmental exposures during pregnancy, such as medication,[5] and infectious diseases,[6] have been suggested as risk factors for isolated oesophageal atresia or for the VACTERL association. A possible association between exogenous sex hormones and OA/TOF or VACTERL association has been suggested.[7-9] However, a number of other studies have not been able to confirm this association.[10,11] So far, no environmental risk factor has consistently been identified.[9-11]

Diethylstilbestrol (DES) is a synthetic oestrogen that was used for the prevention of miscarriage between 1938 and 1975.[12] During this period, it became clear that women who had been exposed to DES *in utero*, that is, women of the second generation, were at increased risk for vaginal and cervical clear-cell adenocarcinoma.[13] Although a recent Cochrane-review could not confirm this association, adenosis of the vagina and cervix and primary infertility were nevertheless found to be significantly more common among women exposed to DES *in utero*.[14]

Experimental animal studies have shown the possibility of transgenerational effects of DES exposure, that is, effects on the third generation, and authors have suggested different hypotheses about the underlying mechanism, including mutagenic effects of DES on germ cells.[15,16] However, data for humans are scarce. So far, the only seemingly strong finding is a significantly increased risk of hypospadias in third-generation males in a Dutch population.[12] This finding however, could not be replicated in a recently published study.[17] Stoll et al. reported congenital deafness in children of mothers exposed to DES *in utero*, but as the authors do not fail to point out, sample size was very small and the association could be coincidental.[18]

In the current study on the aetiology of OA/TOF, a relatively high number of case-mothers was found to be exposed to DES *in utero*. This prompted further research into a potential association between mothers' *in utero* exposure to DES and OA/TOF in their offspring.

MATERIAL AND METHODS

In 2001, a questionnaire was sent to the mothers of children with OA/TOF who were members of the Dutch parents' association of children with OA/TOF (VOKS or Vereniging voor Ouderen en Kinderen met een Slokdarmafsluiting). This is a national support group for parents of children with OA and/or TOF. The six paediatric surgical centers in the Netherlands, to which all children with OA/TOF are admitted based on governmental rules for paediatric surgical practice, are all aware of VOKS and inform parents about it, but contact with VOKS is initiated by the parents.

The questionnaire aimed to identify the specific pre- and postnatal problems that children with OA/TOF and their parents (had) encountered. This questionnaire contained 77 questions about the index-pregnancy, family history, previous and current medical problems and development of the child. One specific question concerned the mother's possible exposure to DES *in utero*. Questionnaires were analysed at the Erasmus MC-Sophia Children's Hospital in Rotterdam as part of an ongoing study on the aetiology of OA/TOF. The researchers verified the questionnaire data on DES exposure by contacting all mothers that mentioned possible *in utero* DES exposure by telephone. If the mothers were sure that their mother had used DES, the answer was interpreted as a certain exposure. If there were doubts about the DES exposure, the medical records of the mother and/or grandmother of the child with OA/TOF were checked, if these were available.

The results of these questionnaires prompted two further analyses. Firstly, the clinical data from all children with OA/TOF that were treated in the Erasmus MC-Sophia Children's Hospital since 1987 were checked. The Sophia Children's Hospital is the paediatric referral center for the Southwestern part of the Netherlands and has the only specialized paediatric surgical intensive care unit in the Netherlands. This area has a population of around four million and approximately 35,000 births per year. Data on all children with OA/TOF treated in the Children's Hospital since 1987 are extracted from the medical records and are available in a comprehensive database. We reviewed this database for any mention of *in utero* DES exposure of the mothers of these children. Possible overlaps with the cases identified from the VOKS questionnaires were accounted for. Informed consent from all participating mothers and institutional approval for the use of the parents' association's questionnaires and for the use of the hospital database were obtained. In those cases in which it was necessary to review the grandmother's

medical records to verify the exposure to DES, informed consent was obtained from the grandmother as well.

Secondly, the data of the EUROCAT registry of congenital anomalies in the Northern Netherlands were reviewed for information about DES exposure in mothers of all registered children with congenital anomalies as well as children with OA/TOF. EUROCAT receives information about DES exposure from standardised questionnaires sent to the parents of all registered children.[19] The EUROCAT questionnaire contains one specific question asking if the mother is a DES-daughter. The medical records are reviewed accordingly.

The incidences of maternal DES exposure for children with OA/TOF and for children with other registered anomalies in the EUROCAT population were compared using Fisher's exact test.

Mothers born before 1947, when DES was first prescribed in the Netherlands, or after 1975, when it was last prescribed, were excluded from the analysis.

RESULTS

Questionnaires were sent to 220 parents of children with OA and/or TOF contacted through the parents' association. A total of 127 (57.7%) completed questionnaires were returned. Three of these concerned mothers born before 1947, leaving 124 mothers for analysis. None of the mothers were born after 1975. Three mothers (2.4%) of children with OA/TOF reported *in utero* exposure to DES.
The Sophia Children's Hospital database yielded 255 cases of OA/TOF since 1987. 18 mothers were born outside the exposure period and there was no information on the dates of birth of 45 mothers, leaving 192 cases for analysis. Six of these children's mothers (3.1%) had reported *in utero* DES exposure, which was confirmed by the medical records in all cases.
There was no overlap between the DES exposed cases of the parents' association and those treated in the children's hospital. The parents' association group included 29 patients that were treated in the Children's Hospital. These were also included in the group of 255 patients registered in the hospital database, as both groups are described separately. None of the mothers of these 29 cases had been exposed to DES *in utero*.

Clinical data of the nine children with OA/TOF born to DES exposed mothers identified through the parents' association and the hospital database are summarised in the table.
None of these cases suffered from intra-uterine growth retardation. Five children were first-borns and six had multiple anomalies, including three cases with VACTERL association. In all mothers DES-exposure was certain, based on medical records of the six cases from the hospital database and on the mother's information in the other three.

Table Clinical history of patients and their study source

Case no.	Sex	Type OA	Associated anomalies	Birth weight (grams)	Gestational age (weeks + days)	Obstetrical history	Family history	Source
1	Girl	OA + TOF	-	2240	36 + 3	G5P2A3	-	P
2	Girl	OA + TOF	Meningocele; agenesis of corpus callosum	2690	37 + 4	G1P1; Later pregnancies: 1 vanishing twin; 1 healthy; 1 Cutis Marmorata Telangiectatica Congenita	-	P
3	Boy	OA	VACTERL association: renal, anal and vertebral anomalies	1470	30 + 4	G1P1	Osler-Rendu-Weber in 2nd degree	P
4	Girl	OA + TOF	Proximally placed thumbs; SUA; mild dysmorphisms; PDA	3315	40 + 2	G2P1A1	-	H
5	Girl	TOF	-	2370	34 + 2	G1P1	-	H
6	Boy	OA + TOF	Unilateral thumb anomaly; mild dysmorphisms	2210	35 + 2	G1P1	Thumb, vertebral anomalies in 2nd and 3rd degree.	H
7	Girl	OA	-	2035	34 + 6	G1P1, IVF	-	H
8	Boy	OA + TOF	VACTERL association: anal atresia, bilateral radial and thumb anomalies, vertebral anomalies	3470	39 + 4	G2P2	-	H
9	Boy	OA + TOF	VACTERL association: anal atresia, unilateral renal agenesis, vertebral and thumb anomalies; SUA; mild dysmorphisms	2300	35 + 4	G3P1A2, subfertility	Spina bifida in 2nd degree. Down's syndrome in 3rd degree.	H

OA: oesophageal atresia; TOF: tracheo-oesophageal fistula; VACTERL: Vertebral, Anal, Cardiac, Tracheo-oEsophageal, Renal, Limb anomalies; SUA: single umbilical artery; PDA: patent ductus arteriosus; IVF: in vitro fertilization; P: Parents' association; H: Hospital database

Between 1981 and 2004, EUROCAT Northern Netherlands registered a total of 8848 children with congenital anomalies (including OA/TOF), whose mothers were born between 1947 and 1975. 33 (0.37%) had a mother who reported *in utero* DES exposure.
Of the 117 cases of OA/TOF ascertained through the EUROCAT registry, 4 (3.4%) had a mother with *in utero* exposure to DES. Exposures were confirmed from the medical records in all 4 cases.
The association between OA/TOF in the child and *in utero* DES exposure of the mother was statistically significant for the data from the EUROCAT registry (P = 0.001, Fisher's exact test).

Specific individual case data of the patients found in the EUROCAT registry were not available. However, we do know that there were three girls and one boy. One child had an isolated OA/TOF and three had associated congenital anomalies. Three cases were first-borns and two children had a birth weight below 2500 grams. One child was conceived after Clomiphene induction, but no IVF or ICSI were used.

There were no overlaps between the EUROCAT cases and the hospital database. However, two of the EUROCAT cases also returned the parents' association's questionnaire and were thus included in both groups. Again, we did not correct for this overlap, as both groups were analysed separately

COMMENT

Our results for the three databases show that, among mothers of infants with OA/TOF, 2.4%, 3.1% and 3.4% respectively, had been exposed *in utero* to DES. To the best of our knowledge, an association between congenital anomalies of the oesophagus and maternal DES exposure *in utero* has not been described before in the literature. Although transgenerational effects of DES have been reported, only anomalies of the genital tract have been described.[12]

The underlying mechanism through which DES might exert its effects on the third generation is unclear.
It is hypothesized that DES causes genetic or epigenetic (imprinting) changes in the primordial oocytes of the mother during her *in utero* exposure to DES.[12,16,20,21] The possibility of a transgenerational effect of hormonal exposure is supported by a recent experimental study of rats which reports that both methoxychlor, an oestrogenic compound, and vinclozolin, an antiandrogenic compound, can have detrimental effects on male fertility of the offspring and that these effects persist until the fourth generation, most likely through an epigenetic change of the male germ line.[22] Other environmental exposures, such as toxic substances or viral infections during pregnancy may modify the effect of DES on specific genes that affect different organs or organ systems.[20,23]

Interestingly, DES has been shown to disrupt the cell cycle of mouse oocytes *in vitro*.[24] This process may also apply to humans, by causing cell cycle disturbances in the development of the oocytes of women exposed *in utero* to DES, thus making the oocytes more vulnerable to detrimental exposures leading to congenital anomalies in the offspring.

Another effect might be that DES induces genetic or epigenetic changes in the somatic cells of the exposed mothers, thereby causing an altered environment that could affect their embryo.[12]

This report describes interesting preliminary data, that have to be interpreted with caution.

Using data from the questionnaires sent to the members of the parents' association may have induced a selection and recall bias. Parents of severely affected patients with OA and/or TOF, i.e. those with multiple associated anomalies, may have been more likely to have joined the association. They may have been more motivated to complete the questionnaire and they may have remembered and mentioned adverse events more easily. Moreover, only a subgroup of 127 parents (57.7%) returned the questionnaire.
However, of these 127 cases, 70 (55.1%) had multiple congenital anomalies and 56 (44.1%) had isolated OA/TOF (no information was available for 1 patient). This is in accordance with previously published studies on OA/TOF, which report that around 50% of cases with OA/TOF have associated anomalies [1,25], suggesting that our population is not very different from the general OA/TOF population. Three of our nine cases (33.3%) had no anomalies other than OA/TOF, making it unlikely that recall bias played a major role. Also, there is evidence to show that recall bias may not be as important as often thought in case-control studies using questionnaire data.[26]

Many DES-daughters have difficulties getting pregnant and some have to use assisted reproductive techniques (ART).[12] There are studies that describe an increased number of congenital anomalies in children conceived by ART (for review, see Hansen, 2005).[27] In our study, IVF was used in one case (no. 7 in the table), subfertility (but no ART) was reported in one case (no. 9 in the table), and Clomiphene (but no IVF/ICSI) was used in one of the EUROCAT cases. Unfortunately, the small numbers and different treatments used make it impossible to speculate on a possible role of ART in the increased risk of OA/TOF observed in our study.

The pattern emerging from the questionnaires strongly resembles that from the EUROCAT data. EUROCAT is a large, well-established birth defects registry with acknowledged methods of case ascertainment and data collection.[19] The information about DES exposure of the mothers of children registered by EUROCAT was obtained from standardised questionnaires. The highly significant association found in the

EUROCAT-population suggests that it is very unlikely that the findings from our questionnaires arose by chance.

In summary, this paper shows a possible transgenerational effect of DES exposure *in utero*. Fortunately, DES is not used for the prevention of miscarriages anymore. However, low-dose environmental chemicals affecting hormone levels may have long-term effects on embryogenesis and fertility of humans exposed to them *in utero* and their offspring.[28,29]

Our study is based on a small number of cases and needs to be verified in a larger study population. Also, the association found does not necessarily imply causality. Both retrospective studies through birth defects registries and (partly) prospective studies through DES registries can play an important role in studying possible associations between DES exposure *in utero* and congenital anomalies in offspring.

In spite of the limitations of our findings, the relatively large number of cases of maternal *in utero* exposure to DES in patients with OA/TOF found in our population should alert us to possible transgenerational effects of DES, related hormones and other environmental factors and underlines the need for continued awareness and follow-up of DES exposed mothers and their children.

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Part V

General Discussion and Summary

General discussion

Chapter 10

GENERAL DISCUSSION

Introduction

Although mortality and complication rates have decreased over the last decades, oesophageal atresia and/or tracheo-oesophageal fistula (OA/TOF) remain severe congenital anomalies that cause significant short- and long-term morbidity.[1-4] There have been many improvements in clinical treatment, such as pre- and postoperative management, anaesthesiology, nursing skills and surgical techniques. In contrast, hardly any progress has been made in understanding the aetiology of these defects. Better insight into processes and factors leading to OA/TOF would enable to develop prevention strategies and would facilitate parental counselling.
This thesis aimed to contribute to the knowledge about the aetiology of OA/TOF by looking at both genetic and environmental factors.

One of the main problems in studying OA/TOF, as well as other congenital anomalies, is that they usually do not present until birth, whereas the developmental defect occurs early in pregnancy, in the case of OA/TOF around the 4th week of gestation. Prenatal care and prenatal diagnostic techniques have strongly improved in the last decades, but prenatal diagnosis of OA/TOF is still very difficult. Indicators are polyhydramnios combined with the absence of a foetal stomach bubble on ultrasound. The sensitivity of prenatal ultrasound in the diagnosis of OA/TOF has been reported to be 42%. In other words, of cases with OA/TOF in whom detailed prenatal ultrasound is performed, 58% is not detected.[5] Choudhry et al. even report 69% undetected cases.[6] A complicating factor is that the foetal stomach bubble is not necessarily absent. In case of a distal TOF or due to gastric secretions, it may be present, but small, making diagnosis much more difficult. As a result, many cases are not suspected at birth and come as a complete surprise to both parents and medical caregivers. Therefore, we are confronted with the presence of a major developmental anomaly around 8 months after the causative events took place.

Genetic Factors

Over the course of the pregnancy, expression levels of both genes and proteins may have changed drastically. Tissue expression levels after birth may therefore not reflect the situation earlier in gestation. However, downstream mediators of faulty genetic processes early in pregnancy might still be measurably present in the tissue, giving us clues to aetiological pathways.
It is of course impossible to collect tissue from the TOF at four weeks. Even if the material were available, for example after a spontaneous abortion, it would be almost impossible to recognise and isolate the different structures. Nevertheless, material from foetuses at intermediate gestational ages, between 15 and 22 weeks, sometimes becomes available after terminations of pregnancy or spontaneous abortions. Pathologists then may find OA and/or TOF, albeit very rare. If there was a spontaneous abortion or intra-uterine foetal death, one should also be suspicious about possible chromosomal or syndromal anomalies, which could be the cause of the death of the foetus. Therefore, tissue from

"normal" OA/TOF – i.e. without a known underlying cause such as a syndrome - is hard to find and was not available for the studies described in this thesis.
In term or near-term patients, material from the TOF can be taken out during surgery. This material is very precious. As most is necessary for oesophageal repair, which of course takes precedence, often only a small piece of tissue can be spared for research. This material can be used to study expression patterns of genes and proteins, as described in **chapter 6**.

A related problem is which tissue to choose for comparison. In fact, there is no "normal" tissue that the TOF can be compared to. In studies looking at changes in a specific tissue type, such as oncology studies, tumour tissue of an organ is obviously compared to normal tissue of that same organ. In the event of TOF, however, determining which tissue to use as a "normal" control is not as straightforward. We chose the two organs probably most closely related to the TOF, namely trachea and oesophagus and a third control, arising from the same embryonic structure (foregut): lung tissue.

When collecting material from control children born at term, it is important to verify that there was no syndrome or chromosomal anomaly and that there is no reason to suspect anomalies of the trachea or oesophagus. "Intermediate age" oesophageal and tracheal tissue can be obtained, but the same criteria apply.
In our studies of gene and protein expression patterns, we used both "intermediate age" and term controls. Unfortunately, very little term control material was available for the gene expression arrays, and quality was low. In the end we were not able to compare our cases to these controls, so we could only make a comparison between (near-) term cases and "intermediate" (very preterm) controls.
It is difficult to say how this could have effected the results of the study. During pregnancy, many developmental processes take place in a relatively short period of time and several stages of development rapidly follow each other. It has been shown in mice that gene expression patterns within one organ of tissue type vary distinctly over different developmental stages[7] Still, it may also be that samples from one specific tissue type will still cluster together, independent of gestational age. That would mean that comparing term TOF tissue with preterm control tissue would give slightly different results than comparing it to term control tissue, but that the main differences between the tissue types (trachea, oesophagus, lung, TOF) would still be visible in the results, as tissue types differ more than do samples of the same tissue type at different gestational ages. The only problem would be similarity of term control samples and fistulas to such an extent that the differences shown between fistulas and controls in **chapter 6** would disappear. However, as these differences are so pronounced, we consider this to be unlikely. Unfortunately, the limited knowledge on the gene expression patterns of these human tissues of different gestational ages precludes drawing definite conclusions about this.
The same problem occurs in other structural congenital anomalies, such as anorectal malformations. This anomaly too presents at birth, but develops early in pregnancy. Gene

and protein expression patterns may have changed during the time in between, for example due to repair or compensatory mechanisms.

This situation of "unideal, but best available" controls, gave us a relatively high *a priori* likelihood of finding differences between cases and controls in the gene-expression arrays (described in **chapter 6**), not related to the pathogenesis of the TOF, but rather to differences in underlying tissue structure that were to be expected because different tissue types were used.
Indeed, the gene expression analysis of tracheo-oesophageal fistulas showed clear differences between cases and controls. All TOFs clustered together and were different from the controls. This shows that the TOF is a distinct tissue type with specific characteristics on a gene expression level, which are shared by all fistulas and which distinguish them from other tissue types.

In addition, there were distinct subgroups within the groups of fistulas. Combining the gene expression data with clinical data of our patients, we were not able to specify clinical characteristics defining those subgroups. Therefore, the significance of the subgroups found based on gene expression patterns is as yet not clear. It may be that these subgroups represent fistulas caused by different aetiologies affecting different developmental pathways, but leading to the same phenotype at birth. Alternatively, a single aetiological factor could affect different developmental pathways, for example when occurring at slightly different time points, leading to the same phenotype of OA/TOF.

As mentioned above, using the "unideal" controls also resulted in a very large number of differentially expressed genes between cases and controls. As described in **chapter 6**, we applied some extra criteria to make the mountain of data manageable.
One such criterion was to only include those genes with a great (3 fold or more, a randomly chosen number) difference in gene expression level between cases and controls. This was on the assumption that great differences in expression level imply a higher likelihood of a causal role for the gene in question. Although this may be the case, it is not necessarily true. A slight change in expression level may have a large impact on expression levels of downstream genes and on developmental processes, as the expression levels of many genes are tightly regulated. Using this criterion therefore, although making our dataset easier to work with, may have resulted in loss of information.
Another approach was looking at genes that were in some way common to all TOFs, in that they either did or did not give a signal in every TOF-sample. This assumes homogeneity and a common pathogenetic mechanism in the TOF group, which may not be the case.
It is hoped that the combination of approaches described above will have led to a picture that is as complete as possible with as little loss of information as possible, but future

studies will have to address these limitations, for example by using larger groups and looking at specific subgroups.

Knockout models

As an alternative for studying human material, OA/TOF can be studied in experimental animals by knocking out specific genes, as described in **chapter 2**, as a way to identify potentially important genes involved in the aetiology of OA/TOF. If knocking out a certain gene causes a phenotype with OA/TOF, the likely conclusion is that this gene is probably involved in the normal development of the foregut. However, extrapolating these animal data to the human situation is very difficult. A good example thereof is Sonic hedgehog (Shh). *Shh* null mutants may have severe foregut anomalies, including OA/TOF.[8] There is only one human patient with OA and a distal TOF in the literature reported to have a deletion of the chromosomal area that contains *Sonic hedgehog*. This child also had growth retardation, microcephaly, eye, limb, skeletal and renal anomalies and a second chromosomal abnormality.[9] However, seeing that many patients are described with deletions of this region who do not have OA/TOF, it is hard to ascribe a causal role in the development of OA/TOF to *Sonic hedgehog* in humans.[10-12] Likewise, it is hard to draw conclusions about the individual contributions of several chromosomal anomalies to the phenotype. *Sonic hedgehog* has been linked to holoprosencephaly, a severe brain malformation, in humans.[13] However, only very few cases of OA/TOF and holoprosencephaly have been reported.[14,15]

Various other genes involved in foregut development cause a phenotype with OA/TOF when knocked out in experimental animals, often combined with severe anomalies of the respiratory system, such as *Thyroid Transcription Factor-1.*[16]

Another, recently described, mouse knockout model for OA/TOF is that of *Nog*, a Bone Morphogenetic Protein (BMP) antagonist. It results in OA/TOF in around 60% of cases. These mice in addition have abnormal morphogenesis of the notochord, similar to that seen in the Adriamycin rat model, which is described in more detail below.[17,18]

As was also recently described, OA/TOF occurs in hypomorphic *Sox2* mutant mice.[19] In humans, mutations and deletions of *SOX2* have been found to cause Anophthalmia-Esophageal-Genital syndrome, which involves OA/TOF.[19,20]

As such, *SOX2* represents one of only two known genes that have been described to cause foregut malformations in both humans and animals, the other one being *GLI3* (discussed in more detail below).

Mutations in other genes that cause OA/TOF in knockout mice have also been described in humans, but none of the patients in the literature was reported to have oesophageal anomalies, again showing the difficulty in translating animal data to human malformations.[21-25]

Teratogenic models

The Adriamycin rat model, which has also been adapted to the mouse, is a teratologic model of OA/TOF.[26,27] Adriamycin is a glycosidic anthracyclin antibiotic, which is used in chemotherapy. When given to pregnant rats during a specific time window in pregnancy, it produces OA/TOF in the offspring.[26] However, no association between Adriamycin or related substances and OA/TOF has been reported in humans.[28-30]

Nevertheless, this animal model is of great value for studies into the embryology, morphology, histology and genetics of OA/TOF. The model produces OA/TOF in a similar pattern to that found in humans, with a proximal atresia with a distal fistula being the most commonly encountered type. Its disadvantage is, that Adriamycin works by interfering with DNA replication and inhibiting DNA and RNA synthesis.[31,32] As such, it will have effects on many more organs than just the oesophagus and it is therefore not surprising that the offspring of rats treated with Adriamycin during pregnancy do not only display OA/TOF, but also other anomalies. Most of these are anomalies of the VACTERL-spectrum, but bladder anomalies have also been described.[33,34] Also, it is not clear what the effects of Adriamycin exposure are on gene and protein expression levels.

An interesting embryopathological theory that arose from studies in this model postulates that the foregut anomalies are accompanied by anomalies of the notochord.[35-39] In the normal situation, the notochord is positioned dorsally from the foregut and separates from it early in gestation, after which the foregut divides into a ventral respiratory and a dorsal oesophageal part. In Adriamycin-treated rats that developed OA/TOF it has been shown to be abnormally ventrally positioned and to remain attached to the foregut longer than usual. In addition, the notochord showed abnormal ventral branching.[36,38,39] Qi et al. speculated that the abnormal position of the notochord may cause traction on the foregut, leading to occlusion of its lumen or even complete interruption and that separation of the notochord from the foregut may be a prerequisite for normal foregut development.[36] Some authors have correlated severity and type of the foregut abnormalities with the length of the abnormally positioned notochord.[39,40] Abnormal development of the notochord has also been linked to vertebral defects, such as hemivertebrae, which are associated with OA/TOF.[38,41-43]
However, as OA/TOF is also found in Adriamycin-treated rats with a normal notochord and as not all Adriamycin-rats with an abnormal notochord develop OA/TOF, the exact nature of the association between these two anomalies is not entirely clear yet.[37]

The studies in the Adriamycin rat model have brought together the teratologic and genetic models of OA/TOF and of the VACTERL association. Other than OA/TOF, as described above, knockout mice for *shh* and its for downstream mediators g*li2* and g*li3* display a range of abnormalities consistent with the VACTERL association.[44,45] The same pattern of anomalies is seen in rats exposed to Adriamycin.[33,34] This observation led to studies into the expression of Shh and its downstream mediators in the Adriamycin model.[46]

One of the roles of the notochord in the development of the foregut is providing a source of signalling activity.[40] The abnormally close position of the notochord to the foregut in the Adriamycin-exposed rats leads to abnormal signalling of Shh, which acts in a dose-dependent way, from the notochord to the foregut.[40,46] This signalling is normally downregulated when the notochord separates from the foregut and this downregulation has been shown to be essential for normal tracheo-oesophageal separation. In Adriamycin-exposed rats, the signalling of Shh from the notochord to the foregut stays high due to the close proximity of the two structures, leading to faulty development of trachea and/or oesophagus.[40,46]

As described above, knockout mice for *Nog* display OA/TOF in around 60% of cases. In addition, they have abnormal notochords, similar to that seen in the Adriamycin rat model, with a prolonged connection and delayed detachment of the notochord to the foregut.[17,18] This could lead to increased signalling from the notochord to the foregut, as described above. Another possible mechanism involved is that of *Nog*-mediated BMP4-inhibition. The notochord normally expresses *Nog*. In *Nog* null mutants there is no inhibition of BMP4 by Noggin, leading to higher levels of BMP4, which promote intercellular adhesion. This might also play a role in the development of OA/TOF.[17]

Li et al. offered a somewhat different hypothesis for the foregut anomalies in their recent study of *Nog* null mutant mice. They observed the same notochord anomalies as described above, but postulated that the imprecise and delayed detachment of the notochord from the dorsal foregut may lead to the notochord taking cells from the dorsal foregut with it as it separates from the foregut. This would then significantly reduce of the dorsal foregut, at that developmental stage composed of a relatively small number of cells, and thereby lead to the formation of an atresia. The prolonged attachment of the notochord to the foregut was explained by increased BMP signalling, which normally would need to be decreased by Noggin for proper detachment to occur.[18]
Disturbances of BMP signalling were also found in TOF samples of humans and Adriamycin-treated rats, adding evidence to the likelihood of involvement of these factors in the development of OA/TOF.[47,48]

Recently, a new mouse model was described, using the mutagen ENU (N-ethyl-N-nitrosourea). In one of the mouse lines produced, phenotypic characteristics of both VACTERL association and caudal regression syndrome were found, with abnormal tracheo-oesophageal separation, anorectal, cardiac, skeletal, limb and renal malformations and pre-sacral mass. The mutation responsible for this phenotype was located to mouse chromosome 19.[49]

Environmental factors and their interactions with genetic factors
There are lines of evidence supporting the idea of combined genetic and environmental contributions to the aetiology of many congenital anomalies, including OA/TOF. For one,

there is evidence of a higher recurrence risk of birth defects in certain families than in the population, but not to the extent that it could be explained by an anomaly of a single gene. Also, monozygous twins have a higher concordance for birth defects than dizygous twins, but this concordance is not complete. Complete concordance would be expected in monozygous twins if only genetic factors were to play a role.[50] Epigenetic factors may also play a role here, as described in more detail below.

Various environmental factors have been implicated in the development of tracheo-oesophageal anomalies, such as exposure to Methimazole, infectious diseases and exogenous sex hormones; so far no single external factor has been pinpointed, although OA/TOF is usually considered part of Methimazole embryopathy.[51-58]

Different environmental factors may affect different genetic pathways, leading to the same phenotype at birth. Alternatively, different environmental factors affect one single genetic pathway, leading to the formation of OA/TOF. Dosing and duration of the foetus's exposure to the environmental factor (medication, teratogens, nutritional factors etc) may also be crucial. Prolonged exposure may lead to building up of concentrations in the foetal system, resulting in damage. Yet, even an acute and brief exposure at a very vulnerable time in development may be enough to cause severe damage.
It has been suggested that it might not so much be the nature of the adverse event but rather the moment of occurrence during pregnancy that will give rise to the anomaly. Different events could cause the same anomaly when occurring during a specific window of susceptibility.[40] Also, other environmental or genetic factors may modify the effects of aetiological factors, thereby influencing the outcome.

Gene-environment interactions have become a focus of research in recent years.[59,60] Individual (genetic) differences in rates of metabolism of toxic substances or nutrients may influence the effects of these substances on the foetus, as these differences may determine the amounts of potentially damaging or necessary protective substances that reach the foetus. This would explain why a specific exposure does not cause the same effect in all foetuses. A good example hereof is provided by studies into maternal folate intake and specific genotypes for a gene involved in folate metabolism in mothers of patients with a cleft lip with or without cleft palate.[61]

There is accumulating evidence that not only mutations in genes (possibly affected by environmental factors) can cause anomalies. Other processes as well, such as posttranslational protein modification by methylation, acetylation or phosphorylation, may be involved in the development of congenital defects.[62] An interesting example in this respect is the finding that a normal cholesterol level is necessary for proper functioning of Sonic hedgehog (Shh).[63] A study in zebra fish showed that foetal exposure to moderate amounts of alcohol alters cholesterol homeostasis, leading to decreased posttranslational modification of the Shh protein by cholesterol, and thus causing faulty Shh signal transduction and developmental defects resembling foetal alcohol syndrome in humans.[62]

Epidemiological approaches

Epidemiological studies of congenital anomalies such as OA/TOF offer a way to study occurrence, trends in frequency, phenotypical appearance, etcetera. In addition, they can shed a light on environmental factors possibly contributing to the aetiology of congenital anomalies.

Questionnaires

For anomalies such as OA/TOF, for which not much is known about possible causative environmental factors, we first need to generate hypotheses about these factors before they can be tested. Studies using questionnaires, such as the one described in **chapter 8**, could provide some insight into the likelihood of certain factors playing a role.

A limitation of such studies may be the effects of recall bias. Mothers of children with anomalies may more easily recall or report adverse events during pregnancy, thereby biasing the results of the study away from the null hypothesis of no difference between cases and controls. Nevertheless, several studies debate the role of such bias and have shown that recall bias, if present at all, would only slightly modify the results of a study.[64,65]

In our studies, we were able to use not only a group of healthy controls, but also the two groups of children with severe congenital anomalies as controls for each other. In these two groups, a similar recall bias would be expected.

A limitation of extensive questionnaires to study potential environmental factors is, that the simple fact that a large number of questions are asked will lead to the finding of random associations based on multiple testing. If a significance level of 0.05 is used, five associations will be found for every 100 questions asked. Therefore, it is important that this type of study is repeated in a different patient population so as to confirm the associations found.

Diethylstilbestrol and other "endocrine modulators"

An interesting issue is a possible transgenerational effect of environmental factors. In **chapter 9**, we describe an association between maternal exposure to the synthetic oestrogen diethylstilbestrol (DES) *in utero* and OA/TOF in their offspring.

The exact mechanism of action of this transgenerational effect is not clear. It is known that DES can alter DNA methylation status and thus may cause aberrant gene expression, which in its turn alters the risk of cancer and other diseases later in life.[59]

DES has also been shown to cause chromosomal aberrations in cervicovaginal tissue of women exposed *in utero*, and to cause changes in gene expression patterns in the mouse Müllerian duct.[66,67]

Thinking along these lines, DES might well induce (epi)genetic changes in the oocytes of the mother during her *in utero* exposure.[59,60,68,69] These changes would then become apparent in the next generation. Effects of oestrogenic chemicals on the oocytes of the developing foetus have been described, i.e. higher rates of aneuploid oocytes and embryos in female mice exposed to Bisphenol A, a synthetic oestrogen, *in utero.*[70]

The effects of DES, or similar substances, might be modified by other environmental factors, such as toxic substances or viral infections during pregnancy, causing disturbances in specific genes that affect different organs or organ systems.[59,71] Individual genetic background may also play a role in the modification of effects of DES.[72]
DES has been shown to disrupt the cell cycle of mouse oocytes in vitro,[73] a process that might occur in humans as well. Cell cycle disturbances in the oocytes of women exposed *in utero* to DES might make the oocytes more vulnerable to detrimental exposures and thus lead to congenital anomalies in the offspring.
Another possible effect of DES is induction of genetic or epigenetic changes in the somatic cells of the exposed mothers, resulting in an altered environment that could affect their embryos.[68]

DES is no longer used for the prevention of miscarriages, but the possible transgenerational effects of this synthetic hormone are nonetheless interesting, as there are many more compounds that could modify endocrine activity. These "endocrine disruptors" or "endocrine modulators" can be man-made (such as phthalates, dioxin and polychlorinated biphenyls) or occur naturally (phyto-oestrogens) and are found in the environment and in many commonly used household products.[74,75] The oestrogenic potential of these substances, the level of endocrine activity that people are exposed to, and the possible effects on (reproductive) health are subjects of debate in the literature.[74-76] A number of studies report changes in the timing of the onset of puberty, decreasing sperm counts, and higher rates of congenital anomalies of the male genital tract in animals and humans and suggest a link between these observations and oestrogenous endocrine modulators in the environment. Although many environmental oestrogens are not as potent as endogenous oestrogens, they are degraded in a slower fashion and they can accumulate in fat tissue. In addition, as humans are at the top of the food chain, they may be exposed to higher concentrations than are animals (for review, see ref. 75). Still, the effects of these endocrine modulators are much debated.[77,78]
Future animal studies in combination with long-term human follow-up studies might shed more light on the effects of exposure to these compounds. They will have to take into account actual exposure levels in humans, endocrine potential, level of exposure relative to endogenous and other exogenous (therapeutic) hormones, the availability of plausible mechanisms of action, and possible confounding factors.[77]

More in general, there are many environmental factors (including nutrients, maternal illnesses, medication, chemicals and radiation) that may influence an unborn child's development, be it through preconceptional mutagenic action on paternal or maternal gametes, or through postconceptional teratogenic mechanisms. Yet, not all foetuses exposed will get the specific birth defect. After an environmental factor is identified, it then becomes interesting to look at the other factors that modify the influence of the environmental factor, thereby determining who is affected and who is not. Examples of such modifying factors are genetic susceptibility or other environmental factors, such as maternal illness.[79]

An important, but often forgotten, player in this field is the placenta. The placenta is crucial in protecting the foetus from damaging environmental influences and in bringing it protecting nutritional factors, oxygen, etcetera. There may be individual genetic differences determining amount and speed of transport of both damaging and protective substances over the placenta, thereby adding to individual susceptibility to birth defects.
Adverse prenatal events may not only damage the foetus, but may also leave their marks on the placenta, giving us clues to the aetiology of the anomalies found in the child. Intra-uterine growth retardation, often associated with placental insufficiency, has been linked to postnatal morbidity.[80] Often disregarded in research into birth defects, the placenta deserves more attention in order to get a complete picture in future research.

Phenotypic expression
In the future, it may be interesting to study specific (more homogeneous) subgroups of OA/TOF, such as cases with isolated OA/TOF, cases with VACTERL association, those with associated cardiac defects, other gastro-intestinal atresias, etcetera, because these subgroups may represent specific aetiologies.
This approach has been used for neural tube defects, in which epidemiological differences were shown between cases with and cases without associated defects thought to be indications of differing aetiologies.[81] In large studies of OA/TOF, only small (if any) epidemiological differences (such as difference in twinning rates, sex ratio, etc.) have been found between cases with and cases without associated defects.[82-84] Nevertheless, for studies into aetiological factors, it may still be interesting to create groups based on the pattern of associated anomalies, as they may represent different aetiologies (that may not necessarily lead to different epidemiological characteristics).

The contributions of different aetiological (genetic and environmental) factors may vary between subgroups. On one end of the spectrum we find the genetic factors leading to a birth defect without any significant environmental involvement. In other cases, genetic factors may provide a background that makes an individual susceptible to the development of a congenital anomaly. A relatively small environmental component superimposed on this background may then be enough for the birth defect to occur. For other patients, we may find environmental factors having a large(r) part in the aetiology. Thus, the relative parts of genetic and environmental factors may differ, while the resulting phenotype remains the same. The concept of one gene, one anomaly - as documented in for example Duchenne's disease[85] - has not been established in patients with OA/TOF, except for a few examples of specific known syndromes, which are discussed below.

VACTERL association
Patients with OA/TOF as part of VACTERL association, as described in **chapter 4**, constitute a specific subgroup of cases with possibly different aetiology as compared with other cases of OA/TOF.

It has been proposed that the VACTERL association should not be seen as an association (defined as: "a non-random occurrence in two or more individuals of multiple anomalies not known to be polytopic field defect, sequence, or syndrome"), but rather as a primary polytopic developmental field defect.[86-88]
A developmental field is defined as "a region or a part of the embryo which responds as a coordinated unit to embryonic induction and results in complex or multiple anatomic structures".[86] During blastogenesis (until the fourth week of gestation), the entire embryo is considered to be one developmental field, the primary developmental field.[89] This later divides into progenitor fields, which form the primordia of all structures of the body.[86] A "hit" during blastogenesis influences the entire primary field, thereby producing congenital defects in multiple organ systems, a so-called polytopic field defect.[86] This mechanism concurs with the above-described idea that it may be not so much the nature, but rather the timing of the prenatal incident that causes a specific pattern of anomalies.[40]
In the case of the VACTERL "association", different adverse events during blastogenesis, such as maternal illness or exposure to toxic substances, may all produce the same end-result. The Shh signalling pathway is a candidate for the developmental pathway that leads to this result. As described above, knockout models of Shh and its downstream mediators display anomalies of the VACTERL-type. In addition, defective Shh signalling has been shown in the Adriamycin rat model, in which VACTERL defects are also apparent. However, as mentioned earlier, a link between anomalies of *SHH* and VACTERL association in humans has not been established yet.
Although there is evidence from animal models that *Sonic hedgehog* might have an aetiological role, a causal role for this gene in the development of OA/TOF in humans has not been proven thus far; therefore, screening all patients with OA/TOF for anomalies of *Sonic hedgehog* is not useful.

Segmental defects
Another subgroup is that of patients with "segmental" defects, such as OA/TOF in combination with vertebral/rib anomalies and possibly cardiac anomalies. These three defects may all occur in the same "body segment" and it is therefore interesting to speculate they represent a subgroup with a specific aetiological basis. Here, there may be a role for the family of *Hox* genes, known to be involved in the segmental patterning of the body.[90] Mice homozygous for a mutation causing a non-functional allele of *hoxc4* were found to display a completely blocked oesophagus and disrupted oesophageal musculature, as well as anomalies of the thoracic vertebrae.[91] Homozygous *hoxa5* mutant mice have tracheal occlusion, abnormal lung and respiratory tract morphogenesis and vertebral anomalies.[92] So far, anomalies of these genes in humans with tracheo-oesophageal abnormalities have not yet been described, and at present it would seem inopportune to screen patients with OA/TOF for anomalies in these genes.

Twins

From an aetiological point of view, the study of twins with congenital anomalies is very interesting. In general, twins have a significantly higher risk of having birth defects, including OA/TOF.[93]

In the population of patients with OA/TOF, twins are at least twice as common as in the normal population.[40,82-84,94,95] Twins can be concordant or discordant for a congenital anomaly. For OA/TOF, most twins are discordant, although a few concordant twins have been reported, both monozygous and dizygous.[40,82-84,95,96] The low concordance rate, even in monozygous twins, supports the idea of a modest contribution of genetic factors to the aetiology of OA/TOF. The genetic makeup of dizygous twins may also cause them to respond differently to environmental factors, possibly causing the discordancy. Although a differential effect of environmental factors may also be an explanation for the discordance in monozygous twins, the study into these factors is very difficult and it has not led to the identification of specific causes so far. Epigenetic mechanisms might play a role here too, as two genetically identical organisms may have a number of differences on an epigenetic level.[97] As an example: there may be differences in methylation of certain genes, causing these to be turned on in the one twin and off in the other, and thus result in phenotypical differences.[98]

The association of twinning with OA/TOF suggests that either the twinning process itself predisposes to the development of OA/TOF and/or that twinning and OA/TOF have one or more risk factors in common that could be the cause of both.[95]

More in detail, there might be a genetic or environmental factor causing the zygotic cleavage and thereby the twin gestation. The cleavage process itself may deprive one twin of a precursor that is essential for development, possibly as a result of asymmetric cytoplasmic delivery during cell division. Deficiency of that precursor may then lead to defective development of the oesophagus in one of the twins. Orford et al. explained the association between twinning and OA/TOF by abnormal notochord morphology. The twinning process may disrupt the organiser from which the notochord arises, thereby causing abnormal notochord morphology and signalling to the foregut, as described above. Another hypothesis assumes that the twinning process causes a global cellular deficiency. The twin with OA/TOF may not recover completely from this deficiency, leading to less cellular material, and thereby a smaller distance, between the notochord and the foregut.[40]

Another explanation is nutritional disadvantage of one of the twins during pregnancy, making this twin more susceptible to environmental insults. Such disadvantage may arise for example from the increased requirements of a twin pregnancy in general, from inadequate placental flow, or from arteriovenous shunting. Unequal blood flow may also cause one twin to receive higher doses of a teratogen or lower doses of a protective nutrient. It could thus lead or predispose to the development of OA/TOF. However, this unequal blood flow would have to occur very early in pregnancy, as OA/TOF develops during the 4th week of gestation. Vascular problems may occur this early in pregnancy,

but they are very uncommon in dichorionic placentas, making this an unlikely explanation for OA/TOF in dizygous twins.[96,99]

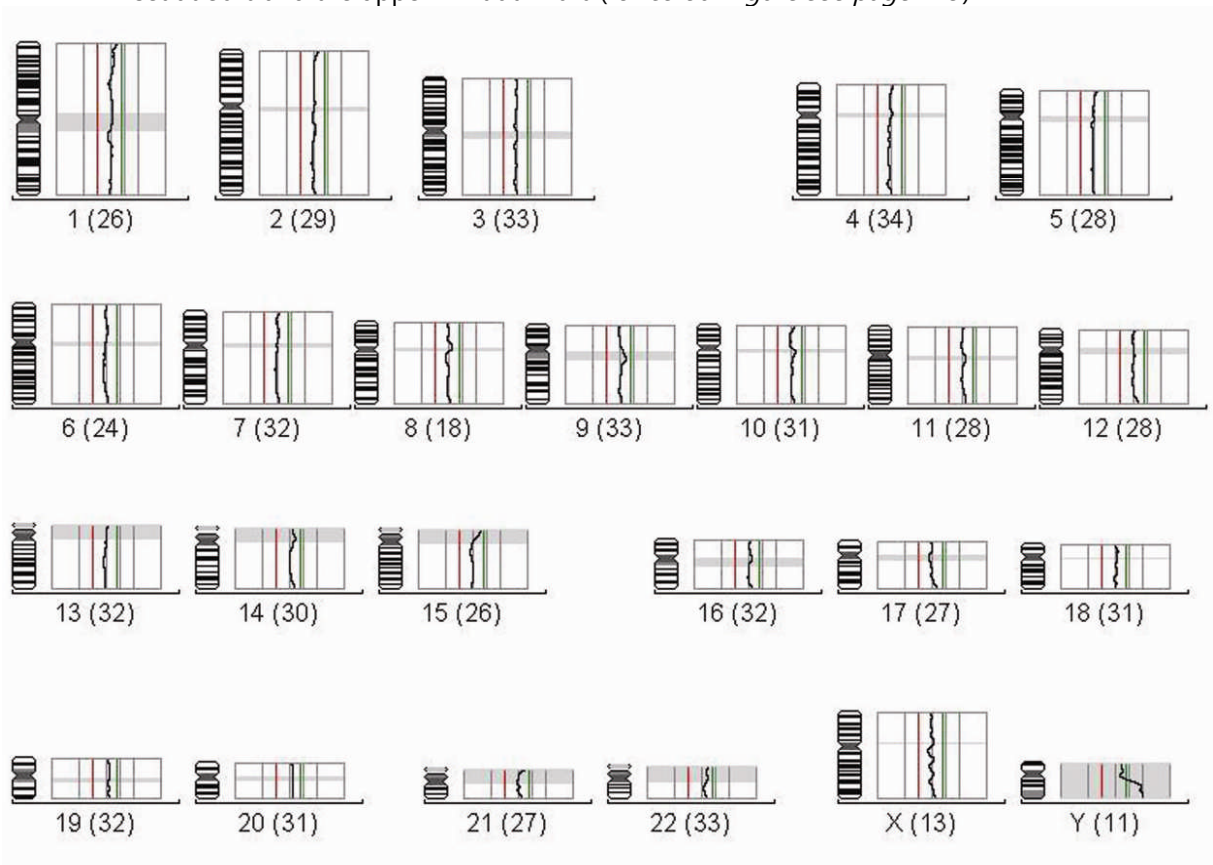
An interesting phenomenon is that of the vanishing twins. Around 30% of pregnancies that start as twin pregnancies reportedly result in singletons.[100] As vanishing twins are not always recognised, the twinning rate in OA/TOF may be underestimated, because those cases are thought to be singleton births. Indeed, the early death of one twin may have been caused by severe congenital anomalies incompatible with life. An thus, the twin with OA/TOF may represent the "better-off" one of the original twins.

Known syndromes
Sometimes, OA/TOF manifests as part of a known syndrome, such as Feingold syndrome. Around 30 - 40% of patients diagnosed with this syndrome have OA/TOF. Mutations in the *MYCN* gene on chromosome 2p24.1 have been shown to cause Feingold syndrome. This allows patients with OA/TOF and other characteristics of Feingold syndrome to be tested for mutations, and to counsel them on the risk to pass the syndrome on to possible offspring. A case of this is described in **chapter 7**. Interestingly, *MYCN* has been shown to be a target of Shh signalling, at least in mouse cerebellum.[101] Although syndromic cases of OA/TOF are rare, studying the specific genetic anomalies involved may provide valuable information about the abnormal processes leading to OA/TOF in these groups.
As described in more detail in **chapter 3**, there are a few other syndromes manifesting OA/TOF for which a genetic basis is known. Deletions and mutations in *CHD7, SOX2* and *MID1* were shown to be a cause of CHARGE syndrome, AEG syndrome and X-linked Opitz syndrome, respectively.[20,102,103] Recently, Que et al. described that hypomorphic *Sox2* mutant mice have OA with a distal TOF, thereby providing a link between animals and humans.[19]

Tracheal agenesis
Embryologically related anomalies, such as tracheal agenesis, as described in **chapter 5**, may share aetiological factors or developmental pathways with OA/TOF. Tracheal agenesis is indeed closely linked to OA/TOF. Both the trachea and the oesophagus develop from the foregut (see **introduction** for an overview). While disturbed development of the foregut will mostly result in OA/TOF, tracheal agenesis as well has been described in the Adriamycin rat model, yet in a small percentage of cases.

Further genetic studies on the six patients described in **chapter 5** and five additional patients with tracheal agenesis have recently been performed. Comparative Genomic Hybridisation (CGH) was normal in nine patients and was uninterpretable in two (Figure 1). More detailed analysis with Multiplex Ligation-dependent Probe Amplification revealed no copy number changes of the telomeric regions in these patients. Further analyses with high-resolution techniques are ongoing.

Figure 1 Example of a normal CGH profile in a patient with tracheal agenesis. The lower limit was set at 80% and the upper limit at 120% (*for colour figure see page 229*)



Synthesis

The aetiology of OA/TOF is heterogeneous and different genes have been implicated in specific subgroups of patients, such as *MYCN* in Feingold syndrome and *CHD7* in CHARGE syndrome.[20,104] Other genes, such as *Sonic hedgehog* (Shh) and *Ttf-1*, have been implicated in mouse knockout models, but corresponding anomalies in humans have not (yet) been found.[8,16] In addition, both in humans and in animal models, imbalances in signalling pathways involved in foregut development, such as the BMP and FGF pathways, have been described.[17,47,48,105] To date, the overlap between genes involved in faulty foregut development in animals and those found in humans is relatively small.

Very recently, OA/TOF was described in hypomorphic *Sox2* mutant mice,[19] providing a link to the mutations and deletions of the human *SOX2* gene that are the cause of Anophthalmia-Esophageal-Genital syndrome, which involves OA/TOF.[19,20] Hypomorphic *Sox2* mice also display severe eye anomalies.[106] Interestingly, in the absence of Sox2 expression, the foregut tube displayed a "tracheal phenotype", with increased 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.[19] The same study showed that Fgf-10 expression decreases *Sox2* expression and increases that of *Ttf-1* in the oesophagus.[19]

Defective FGF signalling has been proposed before as a mechanism involved in faulty foregut development in experimental animals.[105] In the developing mouse foregut, retinoic acid (RA) and Tbx genes are important in the regulation of Fgf10 expression. As described in **chapter 2**, anomalies of the retinoic acid receptors and the *Tbx4* gene have been implicated in the development of OA/TOF in experimental animals.[107-109] Also, in chickens, misexpression of Tbx4 causes ectopic expression of Fgf10.[109] Foxf1, also shown to cause OA/TOF in knockout mice, is a positive regulator of *Fgf10* as well (for review se ref. 110).

Reducing *Gli3* dosage by 50% in a *Gli2*$^{-/-}$ background gives a phenotype of OA/TOF and combined knockouts for *Gli2* and *Gli3* do not form oesophagus, trachea or lungs at all.[111] In humans, mutations in *GLI3* were found to be the cause of Pallister-Hall syndrome, in which OA/TOF is occasionally described.[112-115]

GLI3, as a downstream mediator of Sonic hedgehog, thus provides a link between the well-described Sonic hedgehog pathway in mouse foregut development and the human situation. Immunohistochemistry in one human tracheo-oesophageal fistula could not detect Sonic hedgehog expression.[116] To the best of our knowledge, one case of a deletion of *Sonic hedgehog* and OA/TOF has been reported.[9] However, as mentioned before, it is hard to assign a causal role to *Sonic hedgehog* in this case at present.

Sonic hedgehog has been shown to induce *BMP4* and a number of *Hox* genes during development of chick hindgut.[117] It has been suggested that Hox proteins are downstream proteins in the BMP-pathway.[118] In addition, Shh inhibits Fgf10 expression in the lung. At the same time, Shh regulates the expression of Gli3, which in its turn controls the expression of Foxf1, which then upregulates Fgf10 (for review see ref 110).
As mentioned above, deletions and mutations of the human *MYCN* gene are present in Feingold syndrome. Interestingly, *MYCN* is a direct target of Shh signalling.[101,119]

Knockout mice for different combinations of subtypes of the retinoic acid receptors display abnormal morphology of trachea and oesophagus, in addition to a large number of other anomalies.[107,120] Rat pups born from vitamin A deficient mothers show a highly similar phenotype.[121] During foregut development, RA influences *Hox* genes expressed in the lungs, such as *Hoxa5*.[122] Knockout mice for *Hoxa5* display tracheal occlusion as well as abnormal lung and respiratory tract development.[92] In addition, it has been proposed that RA, through *Hoxa5* and *Tbx4*, influences Fgf10 expression.[122] Knockout mice for another *Hox* gene, *Hoxc4* have a phenotype with OA/TOF and vertebral anomalies.[91]

Mutations in the *MID1* gene are a cause of X-linked Opitz syndrome, in which TOF has been described.[103,123-125] In the establishment of the right-left axis in chickens, Mid1 has been shown to positively influence BMP4 expression and to negatively influence Shh expression. The role of human MID1 in the patterning of the left-right axis is still unclear.[126]

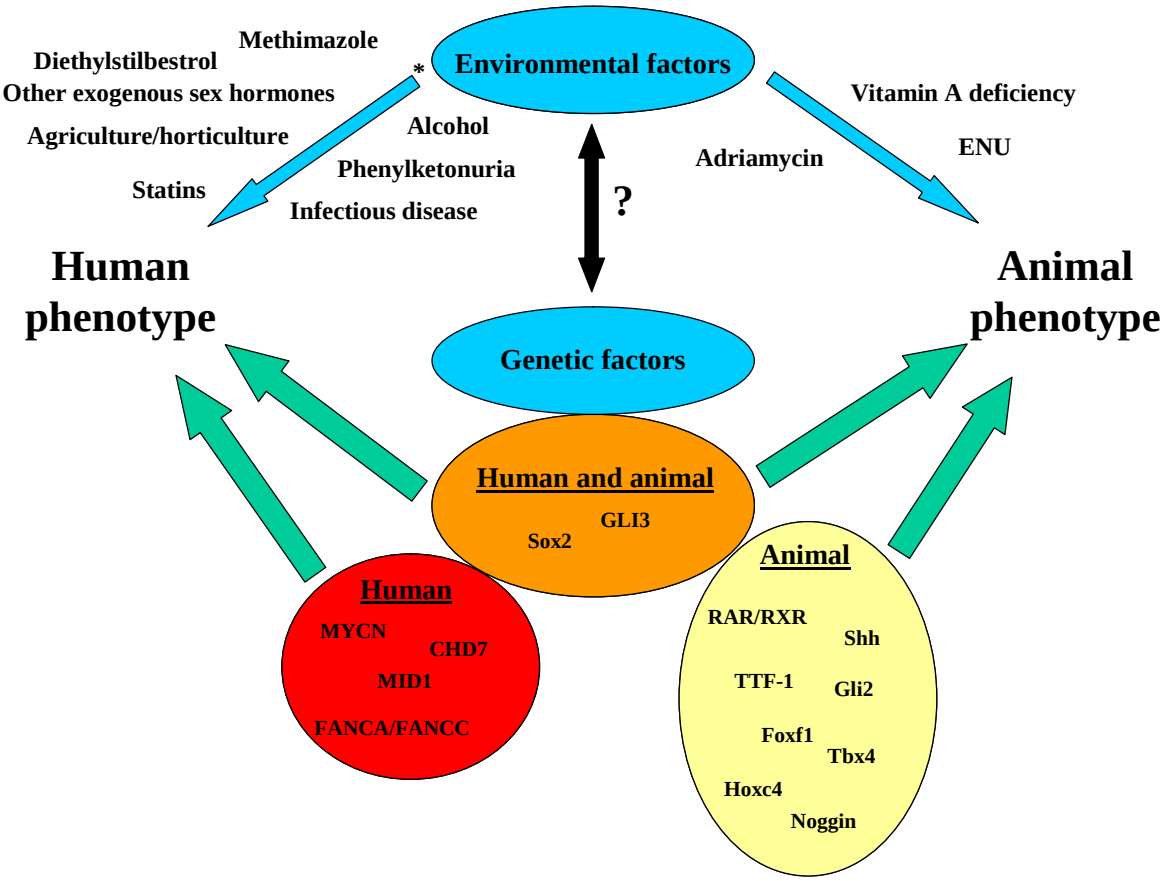
The BMP signalling pathway is a genetic pathway in which disturbances have been described that could lead to the development of OA/TOF.[47,48] However, other than the *Noggin* knockout mice described before, there is no direct evidence for a role of other individual genes in this system. It may be not so much the influence of individual genes, but rather the (im)balance between all system components that determines the direction of foregut development. The same may hold true for other signalling pathways, such as the FGF pathway. In addition, as described above, these pathways are not independently functioning systems. As there are many cross-links between them, imbalances in one system may influence others.
However, as a compensatory mechanism, there is often functional redundancy between different members of a pathway, such as BMP2 and BMP4. If one gene is not functioning properly, its tasks may be partially or fully taken over by one or more other members of the system and dysfunction then need not lead to a phenotype at all.
Alternatively, phenotypic abnormalities caused by an anomaly in one gene may be enhanced when another member of the same pathway is also disturbed.

As described in **chapter 3**, a number of patients with OA/TOF, often combined with other anomalies, show chromosomal anomalies. An interesting anomaly, described in three patients is an interstitial deletion of the long arm of chromosome 17. In, or close to, the deleted region three genes, known from animal studies to be involved in foregut development, are found: *NOG, RARα* and *Tbx4*, discussed in more detail in **chapter 3**. Although these genes are not (always) in the region described to be deleted in humans, their expression may be influenced by the chromosomal anomaly, e.g. through interference with promoter regions.

Various environmental factors have been suggested to be involved in the development of tracheo-oesophageal anomalies, including maternal exposure to methimazole, statins, alcohol or exogenous sex hormones, maternal phenylketonuria and infectious disease and working in agriculture or horticulture.[51-54,58,127-139] For many of these factors, however, a possible role could not be confirmed in other studies. So far, no specific environmental risk factor has consistently been identified.[56,57,140-150]
Our studies implicate a possible role for maternal *in utero* exposure to diethylstilbestrol in the aetiology of OA/TOF in their offspring. The mechanisms for the effects of this external factor, however, have not been elucidated.

In summary, many genes and genetic pathways could be involved in the development of OA/TOF. Some have been proven in humans, some in animals, a few, *GLI3* and *SOX2*, in both. Many of these genes and pathways show interrelations and influence each other. Therefore, disturbances at different levels and in different genes can lead to the same clinical phenotype of OA/TOF. The results of the gene expression arrays described in **chapter 6** support this view, as they revealed many different genes and pathways, mainly those involved in tissue morphology and cellular development.

Figure 2 Factors (possibly) involved in the aetiology of OA/TOF in animals and humans (*for colour figure see page 230*)



*Proven in humans; Proven in humans and animals; Proven in animals; ? possible interactions as yet unknown; * factors with proven or suggested involvement. See text for details.*

A possible influence of environmental factors is certainly not excluded. Still, the mechanisms by which environmental factors may alter development of the foregut, the (epi)genetic effects of these factors and the modifying influences of other external factors are yet to be discovered. In addition, genetic background may affect an individual's response to a certain environmental factor. It is likely that future studies will discover new genes and more relationships between genes, pathways and external factors that we are unaware of at present.
Figure 2 summarizes the current knowledge of the different aetiological factors in OA/TOF.

Registration of birth defects

To create subgroups of patients based on strict definitions of the anomalies involved, an examination of all patients by a clinical geneticist, documenting all defects and dysmorphisms present, and detailed clinical data, including pregnancy history and family history, are needed. Precise documentation of all anomalies may help in diagnosing specific syndromes, and thus aid in counselling the parents or the patients themselves on reaching adulthood, e.g. regarding prognosis and recurrence risk.[151,152]

In addition, databases that store information on all patients with congenital anomalies, including OA/TOF, are pivotal in research into possible causes. Hospital-based or, preferably, population-based birth defects registries are valuable sources of information for many different studies into the epidemiology and aetiology of birth defects, depending on the type of data registered.
Proper registration of congenital anomalies requires a reporting system that is as complete as possible. In general, birth defects registries can either use active case-finding, passive case-finding with verification, and passive case finding without verification. Active case finding involves review of discharge diagnoses of hospitals, followed by gathering more information to verify cases and obtain further details about the cases found. The sources of passive case finding are hospital administrations. Sometimes, cases found through this method are verified to exclude false-positives.[153] The first method of case finding is likely to be the most complete, but it also involves the largest amount of work and resources.

In Europe, a number of birth defects registries have joined forces in EUROCAT, the European network of population-based registers for the epidemiological surveillance of congenital anomalies. EUROCAT covers a quarter of all births in Europe, which comes to around 1.2 million births covered per year.[154] The registries provide valuable information on trends in frequency of occurrence of birth defects and patterns of anomalies seen. One of the objectives of EUROCAT is to facilitate early warning of teratogenic exposures.[154] This international collaboration is especially valuable for rare exposures or rare anomalies, making it possible to combine data and draw more firm conclusions faster, thereby aiding in prevention. Also, the pooling of data enables research questions to be answered on a larger scale, making results more stable.
In themselves, the separate EUROCAT registries also provide data that are readily available for researchers, clinicians and possibly also policy makers.
In the United States, there is a large collaboration between birth defect surveillance systems in eight states, the National Birth Defects Prevention Study (NBDPS). This project covers around 482,000 births annually and is set up as a case-control study, in which mothers of all participating cases and controls are interviewed and buccal swabs are collected from infants and parents for storage and future research.[50] These initiatives offer excellent opportunities for future studies into gene-environment interactions. Of course, these large collaborations require enormous administrative efforts with regard to separate IRB approvals, standardised case definition, study protocols and methodology, protection of identifiable personal data of the study participants, accessibility of the information to different groups of people, such as collaborators, external reviewers and the general public, ensuring correctness of the data, etcetera. In addition, they require a long-term commitment from the participating centres and investigators, as well as ongoing financial resources.
Individual birth defects are often rare, and may involve multiple exposures (genetic or environmental); many pathogenic mechanisms are still to be elucidated and there may be large differences in population prevalences in different areas and occurrence of

specific birth defects among subgroups in the population.[50] Collaborations between centres may provide a means to overcome these difficulties in birth defects research.
Worldwide, birth defects registries collaborate in the International Clearinghouse for Birth Defects.[155]

In the Netherlands, EUROCAT Northern Netherlands registers all children with birth defects born in the provinces of Groningen, Friesland and Drenthe. Detailed information is collected on type of birth defect, associated anomalies, prenatal exposures, delivery and family history.
Chapter 9 discusses the results of a study conducted in collaboration with EUROCAT Northern Netherlands, looking at a specific teratogen, namely exposure of the mother to the synthetic oestrogen diethylstilbestrol (DES) when she was *in utero* herself.
Unfortunately, EUROCAT Northern Netherlands only covers a relatively small proportion of the Dutch population. In the future, it is essential to have a database of congenital anomalies which has national coverage without loss of data quality.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

As there are hardly any families in which more than one member is affected by OA/TOF, large family studies, that could be used for linkage-analysis, are not possible in this group of patients. Genome-wide scanning of patient DNA using techniques such as array-based Comparative Genomic Hybridisation (array-CGH) offers a better approach to identify candidate regions and genes, as has been shown for another major congenital anomaly: congenital diaphragmatic hernia.[156,157]
When an anomaly is found in a patient with OA/TOF, the next step is to study the parents' DNA for the presence of the same anomaly. If so, the likelihood that this anomaly is the cause of the oesophageal phenotype strongly decreases, although the effects of modifier genes and possible epigenetic mechanisms cannot be completely excluded.[158]

Every child with a major congenital anomaly should be seen by a clinical geneticist specialised in dysmorphology and should be included in a follow-up programme which involves medical progress, development and, if applicable, repeated genetic consults to follow dysmorphologies over time, as these may decrease or become more pronounced, providing clues about possible underlying syndromes.
In case of death, efforts should be made to obtain parental consent for autopsy by a paediatric pathologist. This way, all internal anomalies can be documented and a full picture of the child's defects and cause of death can be obtained. In addition, if there is consent to do so, tissue samples can be taken and stored for future studies. If the TOF is still *in situ*, it can be taken out *en bloc* with (part of) the trachea and the oesophagus, so that anatomical relationships and expression of genes and/or proteins in the course of the fistula tract and surrounding tissues can be studied.

If parents refuse consent for autopsy, total body MRI and/or X-rays should be considered. In addition, photographs should be taken of interesting or rare anomalies and dysmorphisms, and measurements taken, for example of facial proportions.

This also calls for detailed registration of cases, involving pregnancy and possibly pre-pregnancy characteristics, family history, clinical data and follow-up. Such registration will provide health care professionals with the information they need to council future parents. Good clinical databases, preferably population-based, can serve as a basis for research into both environmental and genetic causes. National and international collaborations will aid in enlarging the study populations, thereby enabling researchers to draw firmer and more stable conclusions within shorter periods of time.
Subgroups of cases with a specific phenotype can be made based on these databases. In addition, inclusion of data of healthy controls will enable case-control studies. As most birth defects are rare, large prospective studies are hardly possible and case-control studies offer a good alternative.

Collection of biological samples (blood, buccal swabs, urine, etc) will strongly enhance the value of birth defects research. These samples may serve to measure concentrations of environmental substances and micronutrients, but they may also provide information on clinical characteristics, such as infections.[159] After birth, the placenta should be stored for future research. In addition, DNA from the child and possibly the parents can be isolated from blood or buccal swabs and stored for future research. Having a DNA bank combined with detailed patient characteristics offers the big advantage of a readily available stock of DNA that can be used almost instantly when a gene or locus of interest is found. Also, gene-gene and gene-environment interactions can be studied. Information on mutations of a specific gene found in a subpopulation of patients can be added to the database. In later studies, researchers may wish to exclude patients with that specific mutation from their analysis. Alternatively, if a mutation only causes the phenotype of interest when combined with a specific other genetic or environmental factor, researchers may want to study the specific subgroup that has this mutation.[159] DNA banking requires a keen eye for privacy considerations, ensuring limited access to the DNA, proper coding and safe storage.

DNA testing techniques are rapidly developing and enable to detect even smaller aberrations in DNA structure. The interpretation of the results of such DNA tests is not always straightforward. Genetic mechanisms such as variable expression and multiple-gene disorders, in addition to epigenetic mechanisms as described above, may play a role and careful evaluation and confirmation of the results in combination with counselling by clinical geneticists is needed to provide families with the information they need. This will help them make informed decisions about issues like treatment options and future reproduction.
In addition to the genetic data, epidemiological data are needed to gain insight in environmental causes, natural history of an anomaly and prognosis. Only detailed

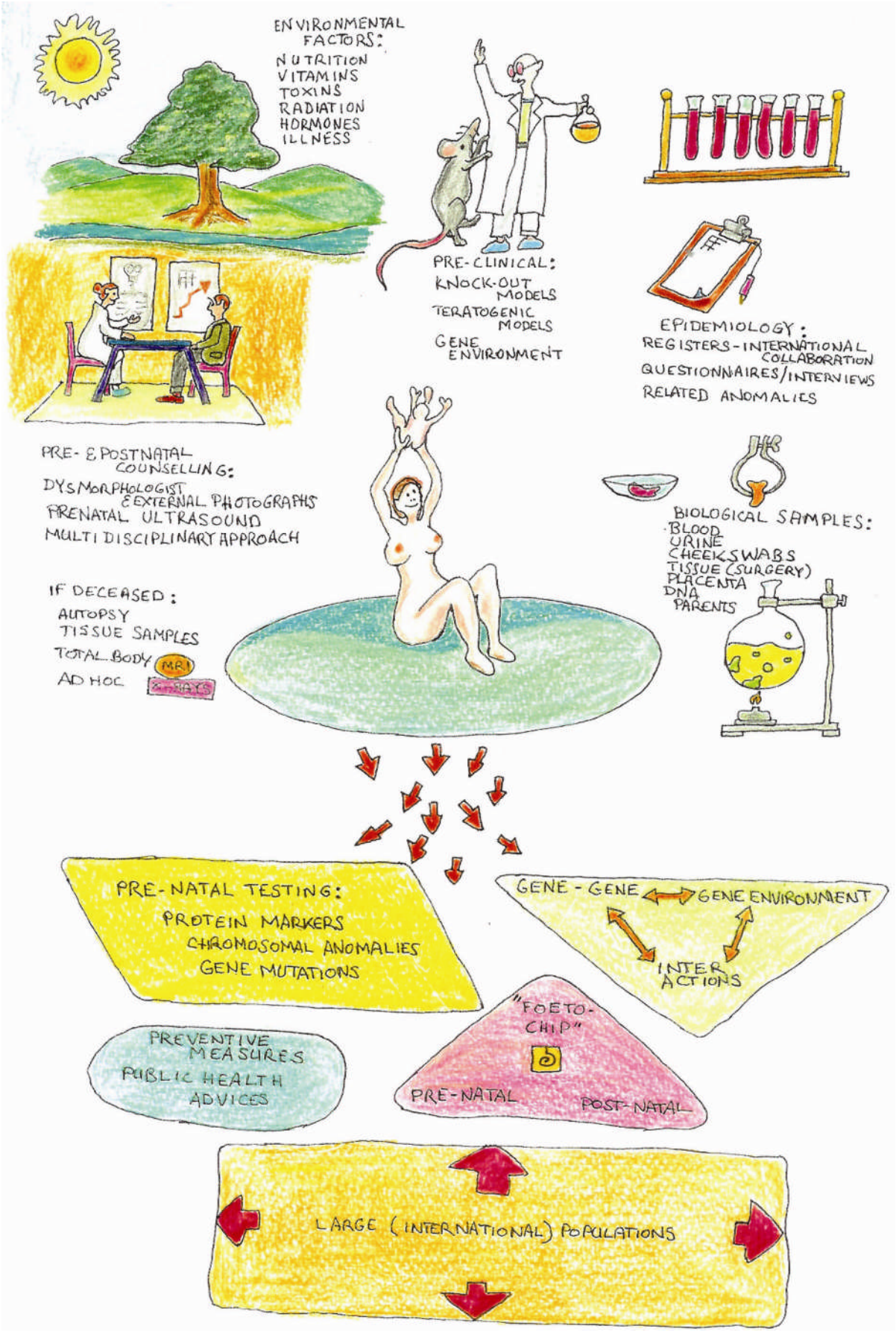
information about type of exposure, exact timing of exposure, other possibly teratogenic exposures or underlying illnesses will lead to a realistic estimate of the effect of a specific teratogen.

These databases, birth defect registries and DNA banks are rich sources of information and provide an excellent starting point for aetiological studies of congenital anomalies. With better aetiological knowledge we can recommend preventive measures, such as the advice to use folic acid in the periconceptional period as a means to lower the risk of neural tube defects in the offspring. In combination with studies on gene-environment interactions, these studies can lead to more targeted prevention strategies for groups or even individuals who are at higher risk because of their genotype. In addition, determining a role of a specific teratogen or (micro)nutrient in the aetiology of one or more birth defects may help in the understanding of the pathogenesis of those birth defects.[160]

Prenatal testing for OA/TOF is now limited to ultrasound scanning, which is not very sensitive. With the exception of mutations in genes present in specific syndromes involving OA/TOF, no causative genetic anomalies are known. More detailed molecular genetic techniques might unveil more genetic anomalies. Genes and/or proteins that are discovered to play a role in the aetiology of OA/TOF in general, of subtypes of OA/TOF, or of OA/TOF in combination with specific other congenital anomalies, could then be used as markers for prenatal testing.
In the future, one could even imagine a "foeto-chip" becoming available: a specific array for prenatal genetic testing for a wide spectrum of congenital anomalies, from the surgical index diagnoses to severe cardiac anomalies, etc. This chip could be used for high-risk pregnancies, such as those with a family history of congenital anomalies and those selected on the basis of prenatal ultrasound examinations. Such a chip could provide important information about short- and long-term prognosis, thereby aiding in parental counselling and preparation of medical care (Figure 3).

In conclusion, the combined efforts of many different specialists, including paediatric surgeons, paediatric intensivists, neonatologists, clinical geneticists, obstetricians, nurses, pathologists, epidemiologists, basic scientists and many more will eventually elucidate aetiological factors involved in the development of congenital anomalies such as OA/TOF. This knowledge can then be used for better treatment, counselling and, in the future, possibly prevention.

Figure 3 Artist's impression by Jacob van der Goot of the aspects of research surrounding a child with a congenital anomaly (*for colour figure see page 231*)



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Summary
Samenvatting

Chapter 11

SUMMARY

Oesophageal atresia with or without tracheo-oesophageal fistula (OA/TOF) is a severe congenital anomaly that affects around 1 in 3,500 births. Not much is known about the causes of this congenital defect. The aetiology is thought to be multifactorial, with both genetic and environmental factors playing a role.
Chromosomal anomalies have been reported in up to 10% of cases of OA/TOF, but no single specific chromosomal defect has been confirmed as an aetiological factor. However, four genes, *MYCN*, *CHD7, MID1* and *SOX2,* have recently been implicated in rare cases of syndromic OA/TOF.
A number of environmental factors have been suggested in the literature to play a role in the development of tracheo-oesophageal anomalies, including maternal exposure to certain medication, alcohol or exogenous sex hormones, or maternal illness. However, many of these factors could not be confirmed in other studies and so far, no specific environmental risk factor has consistently been identified.
It is also still unknown whether genetic factors or environmental factors predominate in the aetiology of OA/TOF. It is likely, that the contributions of these factors differ between (groups of) cases. This thesis aimed to gain more insight in the aetiology of OA/TOF by studying both genetic and environmental factors.

In **Part I** of this thesis, an overview of the subject is given. **Chapter 1** gives an introduction to OA/TOF. The epidemiology and embryology are discussed, as well as what is known from the literature about the aetiology of this congenital defect.
In **chapter 2**, we describe the genes that have been identified in transgenic animals to play a role in the development of the foregut. With one exception, all genes discussed in this chapter give a phenotype of OA/TOF when knocked out.
The chromosomal anomalies that have been reported in patients with OA/TOF in the literature are summarised and discussed in **chapter 3**. Quite a number of chromosomal abnormalities have been described, but no single specific one has been linked to the development of OA/TOF, with the exception of the four genes mentioned above.

Part II describes phenotypic aspects of OA/TOF. In **chapter 4**, patients treated in the Erasmus MC – Sophia Children's Hospital as well as patients from the Paediatric Surgical Centre Amsterdam who had OA/TOF in combination with at least two other congenital anomalies of the VACTERL-type are described. The VACTERL association is the most frequently found association of anomalies in patients with OA/TOF. All congenital anomalies in this specific group of patients are described in this chapter. It was found that of all patients with OA/TOF, 23.5% had at least two additional VACTERL-type anomalies. The vertebrae/ribs and the cardiovascular system were most commonly involved. The most interesting finding was, that a large percentage of cases (70.7%) had additional non-VACTERL-type defects.
Chapter 5 describes a group of patients with tracheal agenesis, a very rare anomaly, which is developmentally related to OA/TOF and can potentially be considered as one

end of the spectrum of foregut anomalies. The group of 6 cases described is the largest case series reported in the literature. We discuss clinical characteristics, classification, embryology, associated anomalies and therapeutic options, although the latter are very limited.

In **Part III**, genetic aspects of the aetiology are described. **Chapter 6** reports the results of a study into the gene expression patterns in the TOF. An unbiased, whole-genome approach using microarray chips was used to screen RNA isolated from TOF samples taken out during surgery. TOFs were compared to tissue samples of normal trachea, oesophagus and lung. We found that the TOFs constituted a clear group that clustered together and could easily be separated from the controls. Within the TOF group, there were at least two subgroups. Based on the number of differentially expressed genes, TOFs were by far the least similar to lung tissue and the most similar to oesophagus. From the analysis of genes that showed significantly different expression between TOFs and trachea and TOFs and oesophagus, a number of important functions arose, including cellular development, tissue morphology and tissue development. In a separate analysis, we also found a number of chromosomal regions with differential expression between TOFs and controls were found. However, a definitive answer regarding the aetiology or pathogenesis of OA/TOF could not be provided.
In **chapter 7**, we present a case report of a patient with clinical characteristics of Feingold syndrome, who was shown to have a hitherto unreported missense mutation in the *MYCN* gene. Multiple of his family members also displayed minor anomalies that could be part of Feingold syndrome, but no mutations were found in these family members, excluding the possibility that they represent variable expression of this syndrome. In addition, behavioural problems were seen in the patient, of which it is uncertain if they should be considered part of the syndrome.

Part IV describes environmental factors in the aetiology of OA/TOF. In **chapter 8**, the results of a questionnaire study, looking into environmental factors in the month before and the first three months of pregnancy, are reported. An extensive questionnaire was given to parents of children admitted to the Erasmus MC - Sophia Children's Hospital with OA/TOF, as well as to parents of children admitted with another severe congenital anomaly, congenital diaphragmatic hernia (CDH), and to parents of healthy control children. For OA/TOF, we found that maternal age was significantly higher than in healthy controls. Also, a borderline-significant association with gardening was found in univariate analysis, which remained borderline-significant in multivariate analysis. For CDH, a significant association with maternal use of alcohol was found. A possible explanation for this association might be the effect of alcohol on the retinoic acid pathway.
Chapter 9 describes a study into a possible association between mothers who were exposed *in utero* to diethylstilbestrol (DES) and their children having OA/TOF. We used data from three sources: questionnaires received from members of the parents' association of children with OA/TOF (VOKS or Vereniging voor Ouderen en Kinderen met

een Slokdarmafsluiting), medical records of patients with OA/TOF from our hospital database and the Northern Netherlands EUROCAT birth defects registry. 2.4 to 3.4% of mothers in all three groups reported *in utero* exposure to DES. We could only do a statistical analysis on the EUROCAT group, which showed a significant association between maternal *in utero* exposure to DES and OA/TOF. Thus, this study reports a possible transgenerational effect of DES exposure in the aetiology of some cases of OA/TOF.

In **Part V**, the results of this thesis are discussed against the background of the literature and all currently known factors in the aetiology of OA/TOF are unified. Moreover, a general concept for research approaches to be used in future patients with major congenital anomalies is suggested, incorporating preclinical and clinical studies. Also, future perspectives for research and possible clinical consequences thereof are discussed (**chapter 10**).

SAMENVATTING

Oesophagusatresie met of zonder tracheo-oesophageale fistel (OA/TOF) is een ernstige aangeboren afwijking die voorkomt bij ongeveer 1 op de 3500 pasgeborenen. Over de oorzaken van deze afwijking is niet veel bekend. Aangenomen wordt dat er een multifactoriële etiologie is, waarbij zowel genetische factoren als omgevingsfactoren een rol spelen.
Chromosomale afwijkingen worden beschreven in wisselende percentages van de patiënten met OA/TOF met een maximum van 10%, maar er is niet een specifieke chromosoomafwijking beschreven in deze groep. Wel zijn er recent vier genen, *MYCN*, *CHD7, MID1* en *SOX2*, beschreven die een rol spelen in de etiologie van zeldzame syndromale vormen van OA/TOF.
In de literatuur worden enkele omgevingsfactoren genoemd die mogelijk een rol spelen in het ontstaan van tracheo-oesophageale afwijkingen. Voorbeelden hiervan zijn maternale blootstelling aan bepaalde medicijnen, alcohol of exogene geslachts-hormonen, of ziekte van de moeder. Veel van deze factoren konden echter in andere studies niet bevestigd worden. Tot nu toe is er geen specifieke omgevingsfactor met een oorzakelijke rol gevonden. Het is ook nog onbekend of genetische factoren of juist omgevingsfactoren een belangrijkere rol spelen in de etiologie van OA/TOF. Waarschijnlijk varieert de bijdrage van deze factoren tussen verschillende (groepen van) patiënten. Dit proefschrift had als doel meer inzicht te verkrijgen in de etiologie van OA/TOF en heeft zich zowel op genetische factoren als op omgevingsfactoren gericht.

In **Deel I** van dit proefschrift wordt een overzicht van het onderwerp gegeven. **Hoofdstuk 1** is een inleiding op OA/TOF. De epidemiologie, embryologie en wat er bekend is over de etiologie van deze congenitale afwijking worden beschreven.
In **hoofdstuk 2** beschrijven wij de genen waarvan in onderzoek met transgene dieren is gevonden dat ze een rol spelen bij de ontwikkeling van de voordarm. Met 1 uitzondering veroorzaken alle genen die in dit hoofdstuk besproken worden een fenotype met OA/TOF wanneer deze genen uitgeschakeld worden in zogenaamde knockout-dieren.
De chromosomale afwijkingen die in de literatuur beschreven worden bij patiënten met OA/TOF worden samengevat en bediscussieerd in **hoofdstuk 3**. Er is een aantal chromosomale afwijkingen bekend, maar het ontstaan van OA/TOF is nog niet geassocieerd met één specifiek gen, met uitzondering van de vier genen die hierboven genoemd staan, die een rol spelen in syndromale OA/TOF.
Deel II beschrijft fenotypische aspecten van OA/TOF. **Hoofdstuk 4** gaat over patiënten die behandeld zijn in het Erasmus MC - Sophia en in het Kinderchirurgisch Centrum Amsterdam. Deze patiënten hadden OA/TOF met nog minstens twee andere aangeboren afwijkingen van het VACTERL-spectrum. De VACTERL-associatie (Vertebrale, Anorectale, Cardiale, Tracheo-oEsophageale, Renale en Ledemaat afwijkingen) is de meest voorkomende associatie bij patiënten met OA/TOF. Alle aanwezige aangeboren afwijkingen in deze specifieke groep patiënten worden in dit hoofdstuk besproken. De resultaten laten zien dat minstens 23.5% van alle patiënten met OA/TOF twee andere

afwijkingen van het VACTERL-spectrum had. De wervels/ribben en het cardiovasculaire systeem waren het meest frequent aangedaan. De interessantste bevinding van dit hoofdstuk was, dat een groot percentage van de patiënten (70.7%) hiernaast nog andere afwijkingen had die niet tot het VACTERL-spectrum behoren.
Hoofdstuk 5 beschrijft een groep patiënten met een agenesie van de trachea, een zeer zeldzame afwijking die vanuit de ontwikkeling gerelateerd is aan OA/TOF en die mogelijk beschouwd kan worden als het ene uiteinde van het spectrum van voordarmafwijkingen. De groep van 6 patiënten die in dit hoofdstuk beschreven wordt is de grootste groep die ooit in de literatuur beschreven is. We bespreken de klinische kenmerken, classificatie, embryologie, geassocieerde afwijkingen en therapeutische mogelijkheden, hoewel die laatste zeer beperkt zijn.

In **Deel III** worden genetische aspecten van de etiologie beschreven. In **hoofdstuk 6** worden de resultaten besproken van een onderzoek naar genexpressie patronen in TOFs. Wij hebben een benadering gekozen waarin het hele genoom werd onderzocht, zonder van tevoren aannames te doen over de betrokkenheid van specifieke genen of delen van het genoom. Uit stukjes van TOFs die verwijderd waren tijdens de hersteloperatie, werd RNA geïsoleerd, dat werd gescreend met behulp van microarray chips. TOFs werden in deze studie vergeleken met normale trachea, oesophagus en long. De TOFs vormden duidelijk een aparte groep die makkelijk onderscheiden kon worden van de controlegroep. Binnen de TOF-groep konden minstens twee subgroepen onderscheiden worden. Op basis van het aantal genen dat differentieel tot expressie kwam, leken de TOFs verreweg het minst op long en het meest op oesophagus. De analyse van genen die een significant verschil in expressie lieten zien tussen TOFs en trachea en tussen TOFs en oesophagus, leverde een aantal belangrijke functies op, waaronder celontwikkeling, weefselmorfologie en weefselontwikkeling. In een aparte analyse hebben wij een aantal chromosomale regio's gevonden met differentiële expressie tussen TOFs en controles. Een definitief antwoord over de etiologie of de pathogenese van OA/TOF werd echter niet gevonden.
Hoofdstuk 7 is een casusbeschrijving van een patiënt met klinische kenmerken van het Feingold syndroom, die een tot nu toe nog niet beschreven missense mutatie had in het *MYCN* gen. Enkele van zijn familieleden hadden ook kleine afwijkingen die mogelijk bij het Feingold syndroom zouden kunnen passen, maar deze familieleden hadden geen mutaties. Hiermee is de mogelijkheid uitgesloten dat zij een variabele expressie van het Feingold syndroom hebben. De patiënt had ook gedragsafwijkingen, waarvan onduidelijk is of ze geïnterpreteerd zouden moeten worden als onderdeel van het Feingold syndroom.

Deel IV gaat over omgevingsfactoren in de etiologie van OA/TOF. In **hoofdstuk 8** bespreken wij de resultaten van vragenlijsten over omgevingsfactoren in de maand voorafgaand aan en in de eerste drie maanden van de zwangerschap. Ouders van kinderen die in het Erasmus MC - Sophia werden opgenomen met OA/TOF of een andere ernstige aangeboren afwijking, congenitale hernia diafragmatica (CHD), en ouders van

gezonde kinderen kregen een uitgebreide vragenlijst. In de groep met OA/TOF was de maternale leeftijd significant hoger dan bij de groep gezonde kinderen. Ook werd er in de univariate analyse een rand-significante associatie gevonden tussen OA/TOF en tuinieren, die rand-significant bleef in de multivariate analyse. In de CHD-groep werd een significante associatie gevonden met het alcoholgebruik van de moeder tijdens de zwangerschap. Een mogelijke verklaring hiervoor zou het effect van alcohol op het vitamine A-metabolisme kunnen zijn.
Hoofdstuk 9 beschrijft een mogelijke associatie tussen moeders die zelf *in utero* blootgesteld zijn aan diëthylstilbestrol (DES) en OA/TOF bij hun kinderen. De data hiervoor kwamen uit drie bronnen: vragenlijsten ingevuld door leden van de oudervereniging voor kinderen met OA/TOF (VOKS, Vereniging voor Ouderen en Kinderen met een Slokdarmafsluiting), medische gegevens van patiënten met OA/TOF uit de database van het Erasmus MC - Sophia en de registratie van aangeboren afwijkingen in Noord-Nederland, EUROCAT. In alle drie de groepen was 2.4 tot 3.4% van de moeders *in utero* blootgesteld aan DES. Statistische analyse was alleen mogelijk in de EUROCAT-groep. Hierin werd een significante associatie aangetoond tussen maternale *in utero* blootstelling aan DES en OA/TOF. Dit onderzoek laat dus een mogelijk transgenerationeel effect zien van blootstelling aan DES in de etiologie van een aantal patiënten met OA/TOF.

In **Deel V** worden de resultaten uit dit proefschrift bediscussieerd tegen de achtergrond van de literatuur en worden alle op dit moment bekende factoren in de etiologie van OA/TOF samengevat. Hiernaast wordt een algemene benadering voorgesteld voor onderzoek van toekomstige patiënten met ernstige aangeboren afwijkingen, waarbij preklinische en klinische studies worden gecombineerd. Ook worden toekomstige mogelijkheden voor onderzoek en mogelijke klinische consequenties daarvan besproken (**hoofdstuk 10**).

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De medewerkers van het histologie- en immunolab bij de pathologie en vooral Pieter, Frieda en Luc. Dank jullie wel voor de uitleg en het snelle snijden, plakken en verwerken van de coupes

Sigrid Swagemakers en Prof. dr. P. van der Spek, dank jullie wel voor de hulp bij de analyse van de expressiedata en voor het meedenken over het project.

Rory Morty and Oana Amarie, thank you so much for doing the stainings in Giessen, for participating in this project and for your very valuable advice!

Alle genetici en in het bijzonder Yolande van Bever, Alice Brooks, Jeanette Hoogenboom en Lutgarde Govaerts wil ik bedanken voor hun aandeel in het maken van syndroomdiagnoses en het verzamelen van materiaal.

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Margo Terlouw. Heerlijk dat jij de lay-out van het boekje hebt kunnen en willen doen. Jouw nauwkeurigheid, de prettige communicatie en je goede ideeën hebben voor een prachtig resultaat gezorgd, dank je wel! Vera, dank voor je hulp bij de kaft!

Annemarie Illsley en Judith Visser, dank jullie wel voor alle praktische hulp en de gezellige gesprekken. Gaan we snel eten?

Marieke van Dooren, dank je wel voor het opzetten van het onderzoek, zodat ik op een rijdende trein kon stappen!

Liong-Kie Ko, Tanneke Marijt en Joke van Marion van Sanquin Bloedbank dank voor jullie flexibiliteit die mij de mogelijkheid heeft gegeven ook bij de bloedbank te werken.

(Oud-)kamergenootjes Jessie, Renata, Mark (dododidodo), Joke, Sebas, Annelies, Ada, Harma: dank jullie wel voor de gezelligheid! Sandra, het was gezellig om een belangrijk deel van het traject naast je te zitten! Joanne, dank je wel voor lachen en huilen, voor het filmpje, voor foute muziek, voor praktische hulp en voor alle gezelligheid! Ilse, aan het einde van mijn traject kwam jij de kamer versterken en wat werd het gezellig!

Andere (oud-)onderzoekers: Merel, min of meer parallelle projecten, pieper-rooster met Maaike en vooral een nieuwe vriendschap die me veel waard is. Maaike, stukje van jou bij mij in de diepvries of andersom….dank je wel voor je vriendschap, voor de gezellige kopjes thee en het samenwerken. Heleen, dank je wel voor het "even binnenlopen", voor het positief en negatief afreageren, voor je humor en je gezelligheid! Freek, buuffie, we konden bijna naar elkaar zwaaien aan de Boompjes. Dank je wel voor de glaasjes port met blauwe kaas en voor kaasfondue. Even langs gaat niet meer zo makkelijk, maar we gaan gewoon met de trein! Liesbeth, heel veel succes met het voortzetten van het onderzoek, ik ben heel benieuwd hoe het verder gaat.
Irene, Manon, Sinno, Emile, Marloes, Barbara, Maaike R, Niels, Frans, Chris, Marjolein en alle anderen: dank voor de gezelligheid.

Vrienden/friends. Alyde en Niek, wat is het toch altijd gezellig om even bij jullie langs te gaan in Utrecht. Laten we snel weer eten. Clubbies, de laatste tijd vooral emailcontact, volgens mij wordt het weer eens tijd om elkaar te zien. Sarah, de vriendschap met jou is altijd weer even vertrouwd.
Rod Hunt – Our email contact really brightened the days! Cath, we always just pick up wherever we left off. Thank you for sharing your house with me when I was in Sydney. Paul, Glenys, Natasha, Seonaid, thank you for the lovely times in Oz!

Dear Nadia and John, you gave me a home in Sydney and showed me the basics of research – which started my interest in doing a PhD. Nadia, you are a role-model and a great friend. I admire your strength, your professionalism and your kindness.

Carol Bower, my Perth-home-away-from-home. Thank you for your advice, health muffins, M*A*S*H, outdoor cinema and your lovely emails and letters. You are an inspiration.

Nora en Cordula, de afgelopen anderhalf jaar hebben ons dichter bij elkaar gebracht. Ik vind dat we het heel goed gedaan hebben en ben jullie dankbaar voor alle telefoontjes, etentjes en steun.

Matthias. We started in French, continued in English and now we add in some German. You and your family are very special people. Thank you for phone calls, for cups of tea and for walks and talks. Nicki, yoyoyo.

Omi, ik heb bewondering voor u, heerlijk dat u er vandaag bij bent.

J&J&A&F&L&M&P. Zondagavond – family dinner is een traditie geworden. Wat heerlijk dat het bij jullie altijd gezellig is. Oom J, ik vind het heel bijzonder dat jij de laatste tekening voor in mijn boekje gemaakt hebt. Dank je wel.

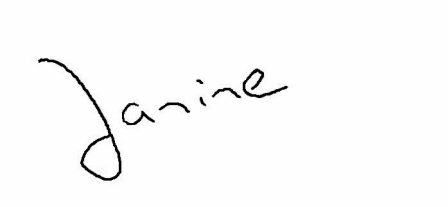
Mijn paranimfen, niet voor niets staan jullie vandaag naast mij!

Ilou, mijn kleine zusje, maar al helemaal niet zo klein meer! Dank je wel voor je humor, je echtheid, je hoofd en je hart. Rogier, gezellig om jou in de familie te hebben.
Maryse, mijn oudste vriendinnetje. Dank je wel voor veel telefoneren, voor het extra dubbeltje, voor gezellig eten en thee drinken, voor snel emailcontact, voor weekendjes weg, voor al je adviezen en wijsheid.
Maarten, collega! Van bijna-Belg werd je echt Belg en toen weer gewoon Hollander. Dank je wel voor je nuchterheid, je humor, voor pizza eten en voor onzinnige emails, voor je advies en je steun.

Pappie. Toen jij er vorig jaar ineens niet meer was, realiseerde ik me hoe hard ik je eigenlijk nog nodig heb. Zie je me?

Mam. Ik kan heel veel tegen je zeggen, maar eigenlijk is het heel simpel. Zonder jou was het gewoon niet gelukt. Dank je wel voor alle telefoontjes, kopjes thee, bloemen, koekjes en voor je steun en je onvoorwaardelijke liefde.

Irwin, lief. Je hebt me gevraagd niet te veel over jou te schrijven. OK, dan alleen het belangrijkste: ik lief jou. Probleem opgelaten. ☺



Curriculum vitae

Janine Felix was born on the 20th of November, 1976 in Rotterdam, the Netherlands. After completing grammar school at the Erasmiaans Gymnasium in Rotterdam, she started medical school at the University of Groningen in 1995. During her studies, she spent a total of 11 months in Australia to do research in the Department of Neonatology of the Children's Hospital at Westmead (Sydney) and the Institute for Child Health Research in Perth. There, she was involved in research projects entitled "Birth Defects in Children with Newborn Encephalopathy", "Autism following a history of newborn encephalopathy, more than a coincidence?" and "Cerebral palsy following term newborn encephalopathy: a population-based study", under the supervision of clinical associate professor N. Badawi, clinical professor C. Bower and doctor J. Kurinczuk. For the presentation of the latter project, she received a young investigator award at the conference of the Perinatal society of Australia and New Zealand in 2004.
After finishing medical school in 2002, she worked as a paediatric resident at the Isala Clinics in Zwolle, the Netherlands.
In March 2003, she started working as a research physician in the Department of Paediatric Surgery of the Erasmus MC - Sophia Children's Hospital on the project entitled "Aetiological studies in oesophageal atresia/tracheo-oesophageal fistula" under the supervision of Prof. dr. D. Tibboel. This research was financially supported by the Edgar Doncker Foundation (Stichting Edgar Doncker Fonds). The work done during this period is presented in this thesis.
In September 2004, she started working as a physician at Sanquin Bloodbank in Rotterdam for a few hours a week.
In August 2007, she has started a Master's degree in Epidemiology at the Netherlands Institute of Health Sciences in Rotterdam.
She lives together with Irwin Reiss.

List of publications

Felix JF, Keijzer R, Van Dooren MF, Rottier RJ, Tibboel D. Genetics and developmental biology of oesophageal atresia and tracheo-oesophageal fistula: lessons from mice relevant for paediatric surgeons. Pediatr Surg Int 2004; 20: 731-736.

Felix JF, Tibboel D, De Klein A. Chromosomal anomalies in the aetiology of oesophageal atresia and tracheo-oesophageal fistula. Eur J Med Genet 2007; 50: 163-175.

De Jong EM, Felix JF, Deurloo JA, Van Dooren MF, Aronson DC, Torfs CP, Heij HA, Tibboel D. Non-VACTERL-type anomalies in patients with oesophageal atresia / tracheo-oesophageal fistula and full or partial VACTERL association. Submitted.

Felix JF, Van Looij MAJ, Pruijsten RV, De Krijger RR, De Klein A, Tibboel D, Hoeve LJ. Agenesis of the trachea: Phenotypic expression of a rare cause of fatal neonatal respiratory insufficiency in six patients. Int J Pediatr Otorhinolaryngol 2006; 70: 365-370.

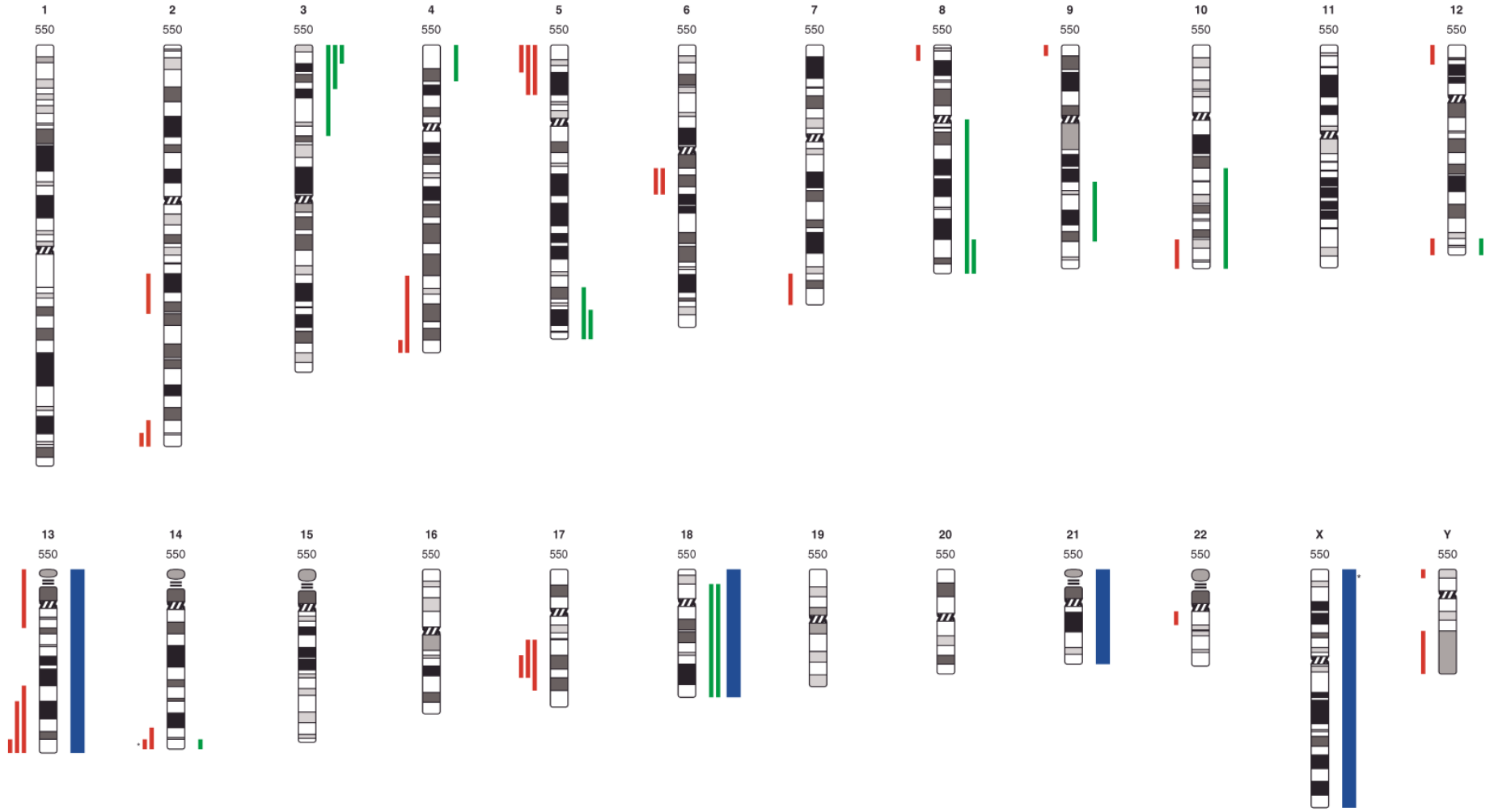
Felix JF, De Brouwer APM, Nabuurs SB, Van Bokhoven H, Tibboel D, Brooks AS. A new missense MYCN mutation in a child with Feingold syndrome. Submitted.

Felix JF, Van Dooren MF, Klaassens M, Hop WCJ, Torfs CP, Tibboel D. Environmental factors in the aetiology of oesophageal atresia and congenital diaphragmatic hernia: Results of a case-control study. Submitted.

Felix JF, Steegers-Theunissen RPM, De Walle HEK, De Klein A, Torfs CP, Tibboel D. Esophageal atresia and tracheoesophageal fistula in children of women exposed to diethylstilbestrol in utero. Am J Obstet Gynecol 2007; 197: 38.e1-5.

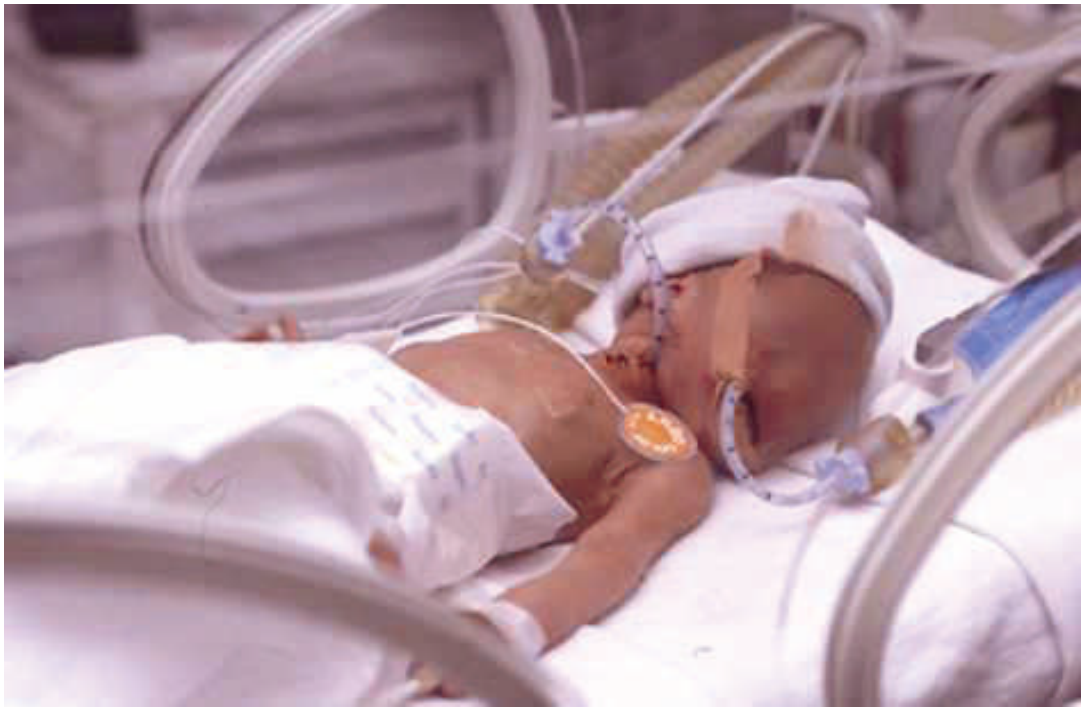
Colour figures

Chapter 3 Figure 1 (*see page 41*) Chromosomal anomalies described in patients with OA/TOF



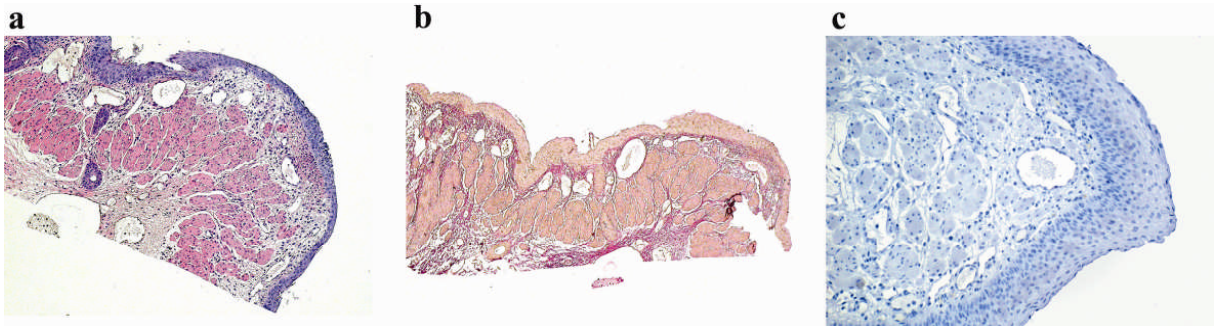
*Deleted regions are depicted in red to the left of the chromosome, duplicated regions in green to the right of the chromosome. Blue bands to the right of the chromosome denote trisomies. Single cases are depicted as single narrow bands. Three or more cases with the same anomaly are shown as broad bands. Triploidy (2 cases), translocations and cases with unspecified breakpoints are not shown. MYCN, SOX2, CHD7 and MID1 denote specific genes in which deletions and/or mutations have been described in patients with OA/TOF (for review see ref. 11 and 12). See text for details. *Mosaic in 1 case.*

Chapter 5 Figure 1 (*see page 77*)



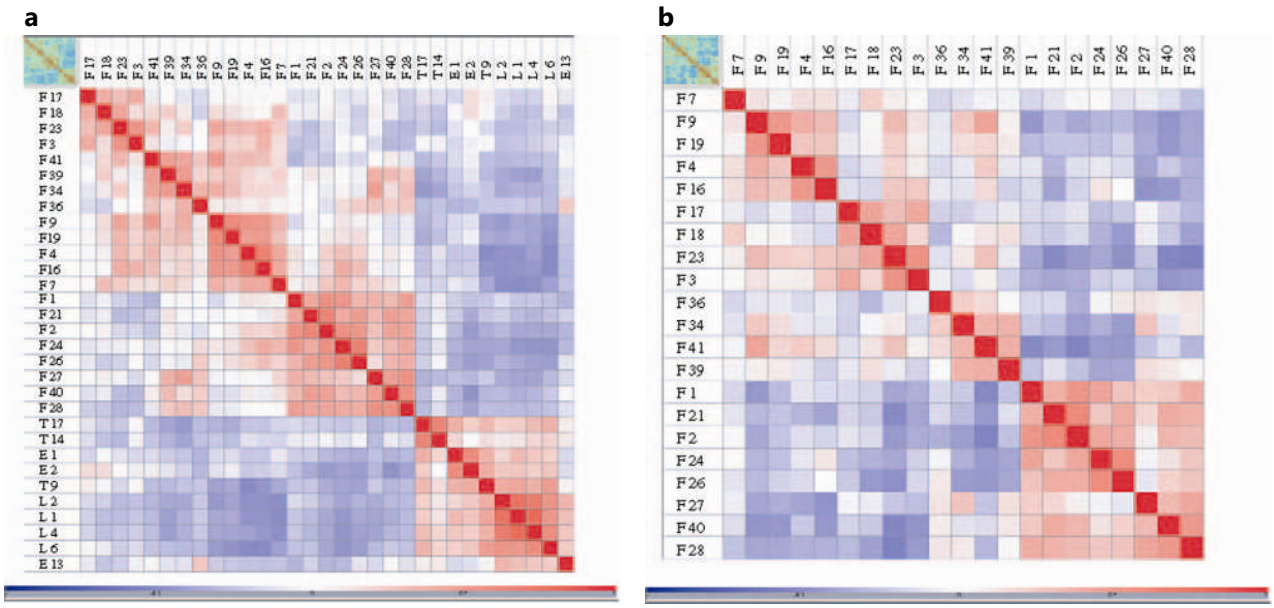
Case no.2, ventilated through two tubes, one tube was inserted orally and the other through the tracheostomy.

Chapter 6 Figure 1 (*see page 92*)



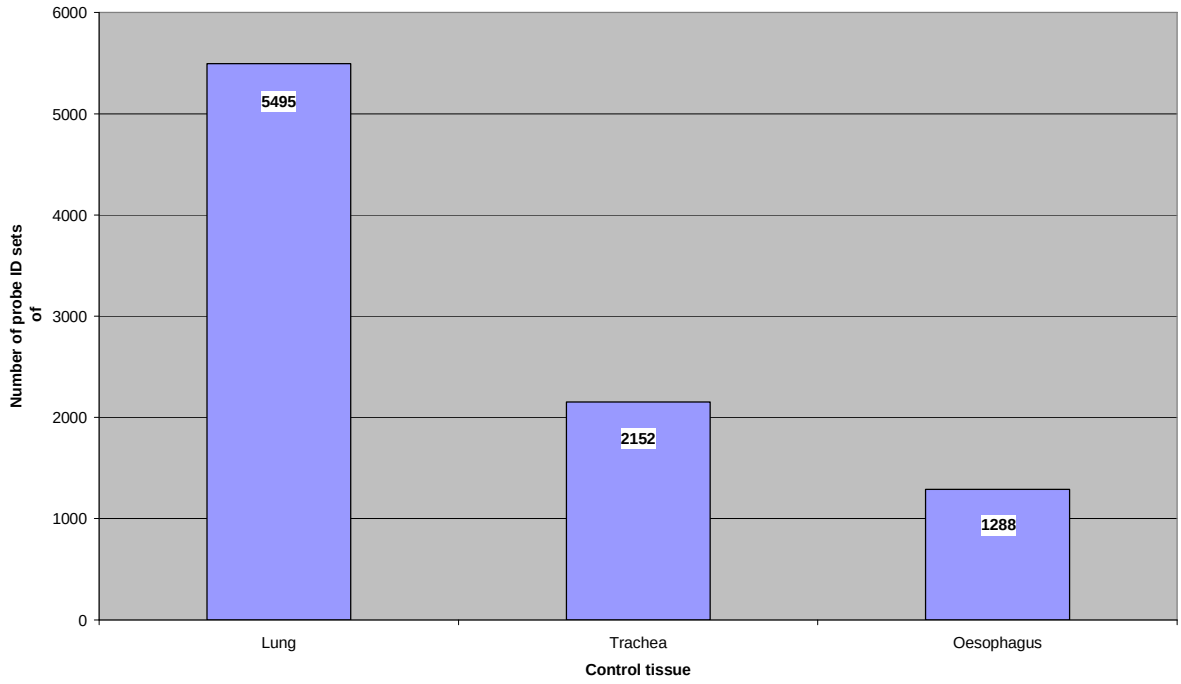
a *Hematoxylin and eosin staining (10x),* ***b*** *Elastica von Gieson staining (5x) and* ***c*** *TTF-1 immunohistochemical staining (20x) of representative samples of the tracheo-oesophageal fistula (see text for details).*

Chapter 6 Figure 2 (*see page 93*)



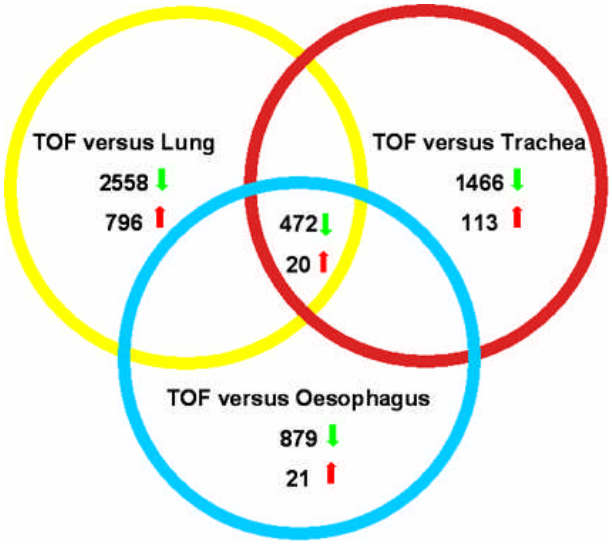
*Correlation plots of all case and control samples (**a**) and of all TOF samples (**b**), including only those genes that showed at least a 2-fold change in expression level compared to the geometric mean in at least one sample. Samples are plotted against each other and the level of similarity of gene expression patterns is determined. Red: high similarity; Blue: low similarity (grading scale, see scale bar at bottom). F: TOF; T: trachea; E: oesophagus; L : lung. Numbers represent sample numbers.*

Chapter 6 **Figure 3** (*see page 95*) Bar chart showing the number of probe ID sets on the gene expression array that showed a significant difference in expression level between the TOFs and the three different control groups



Chapter 6 Figure 4 (*see page 101*)

a



b

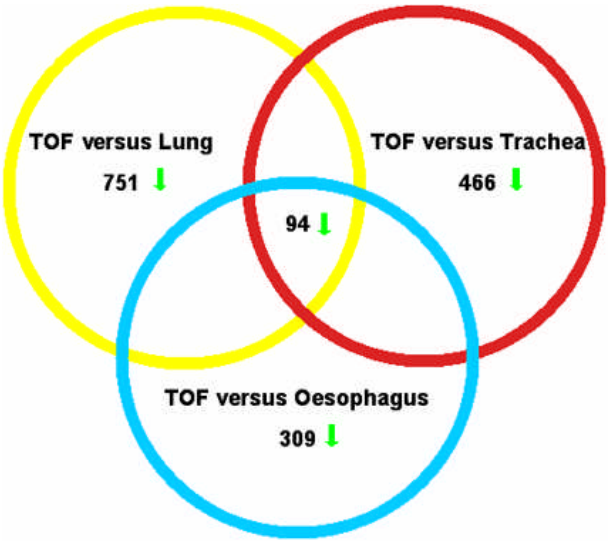
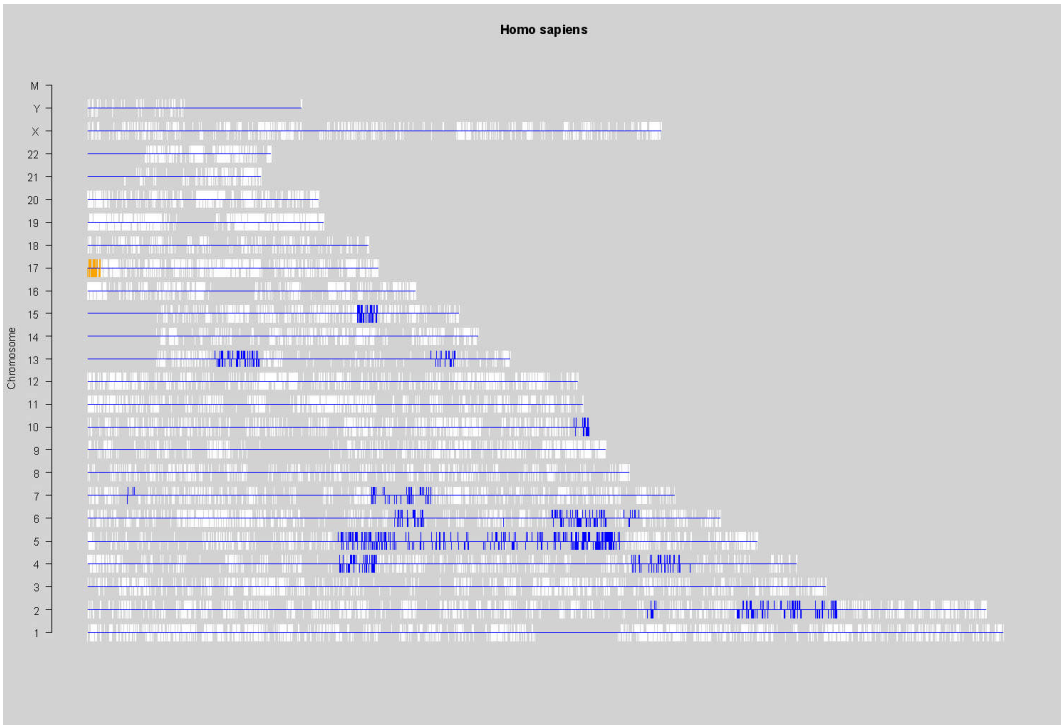


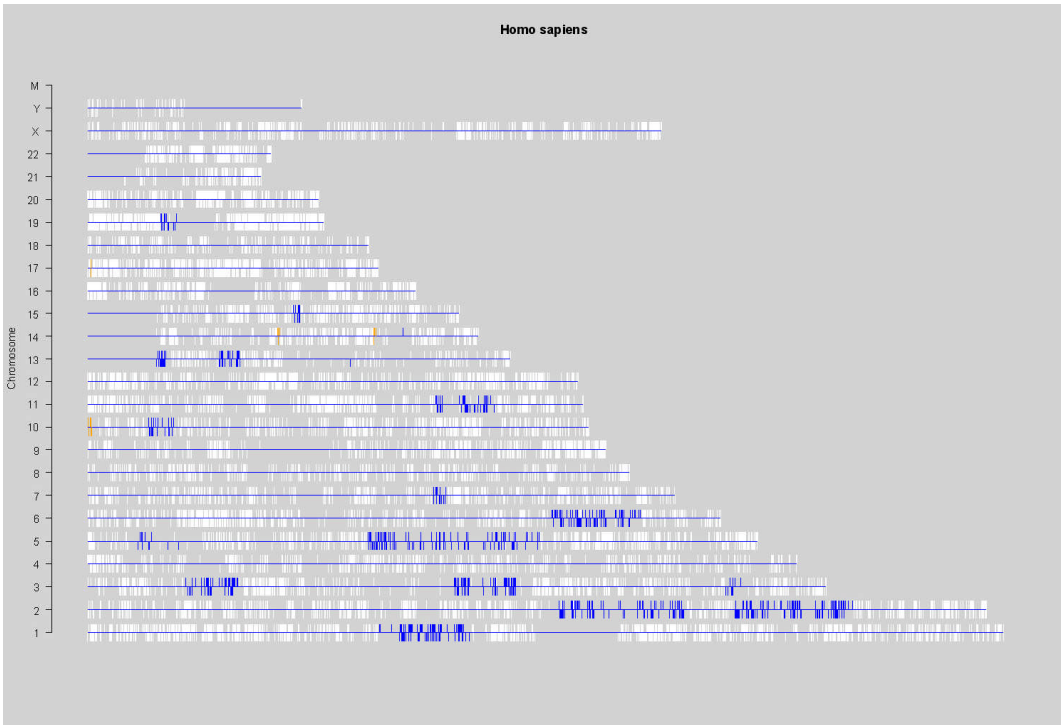
*Diagram showing the results of the SAM analyses of TOFs versus the three control groups including only those genes that were present in all 21 TOFs and the intersection of these analyses (**a**) and including only those genes that were absent in all 21 TOFs and the intersection of these analyses (**b**).*

Chapter 6 Figure 5 (*see page 104*)

a

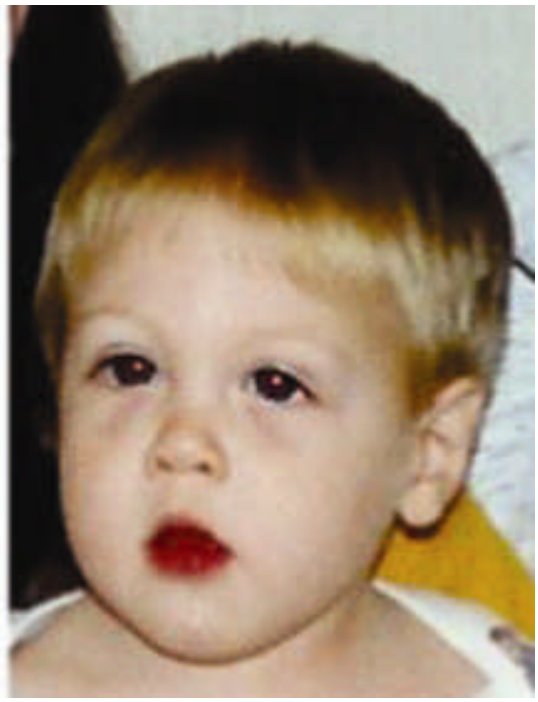


b

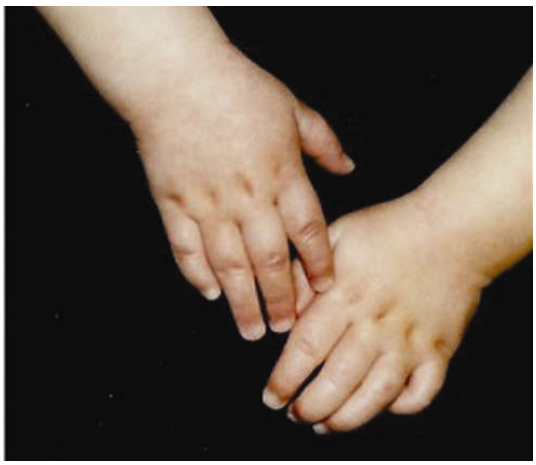


*Results of the LAP analysis showing significantly up- (orange) or downregulated (blue) chromosomal loci in TOFs compared to trachea (**a**) and oesophagus (**b**). A cut-off P-value of 0.05 was used for significance. Chromosome numbers are shown on the left-hand side of the picture. Chromosomes are shown as bars with the p-arm on the left-hand side and the q-arm on the right hand side. White vertical lines depict non-significantly different areas.*

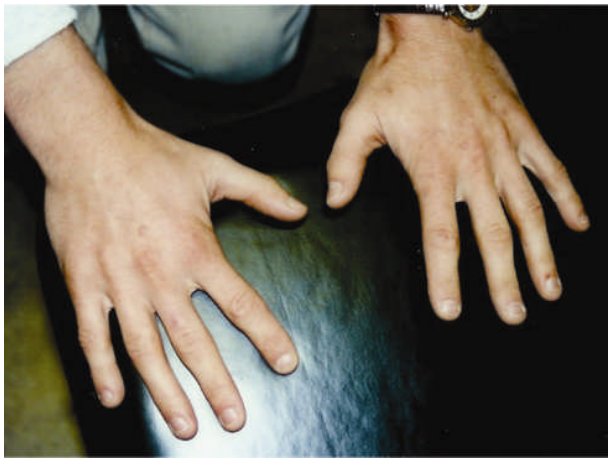
Chapter 7 Figure 1 (*see page 117*) Clinical pictures



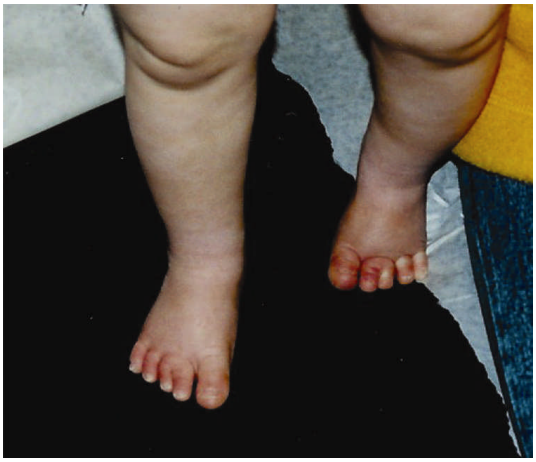
a *the proband's face*



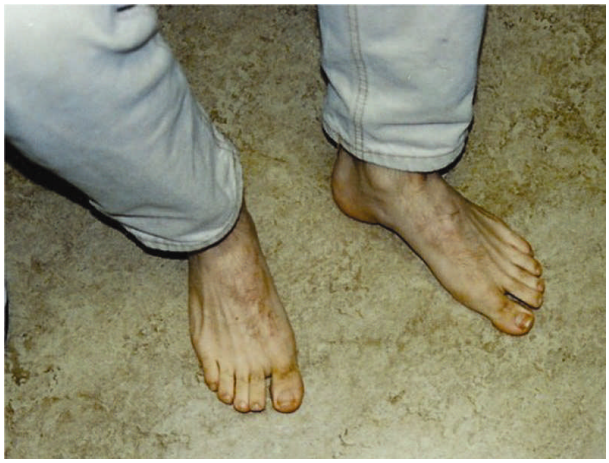
b *the proband's hands*



d *the father's hands*

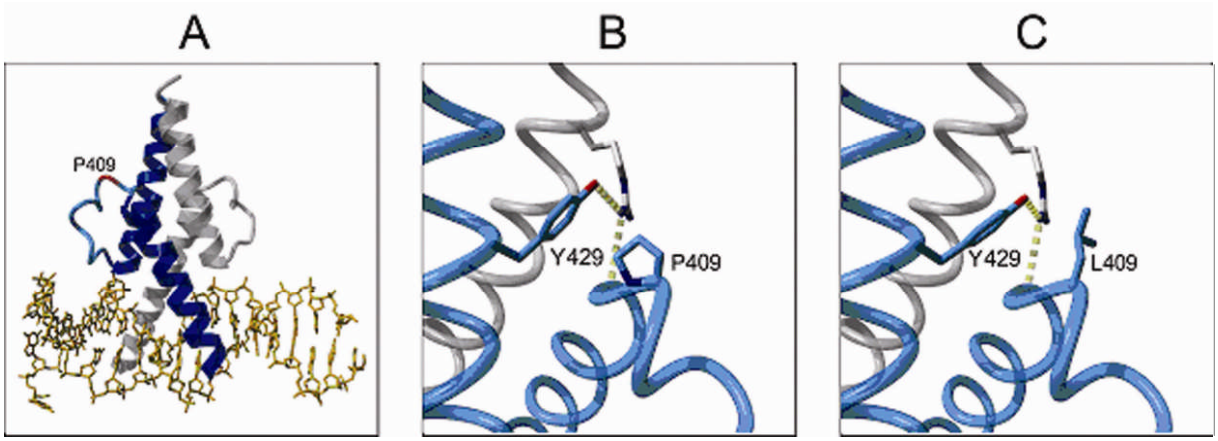


c *the proband's feet*



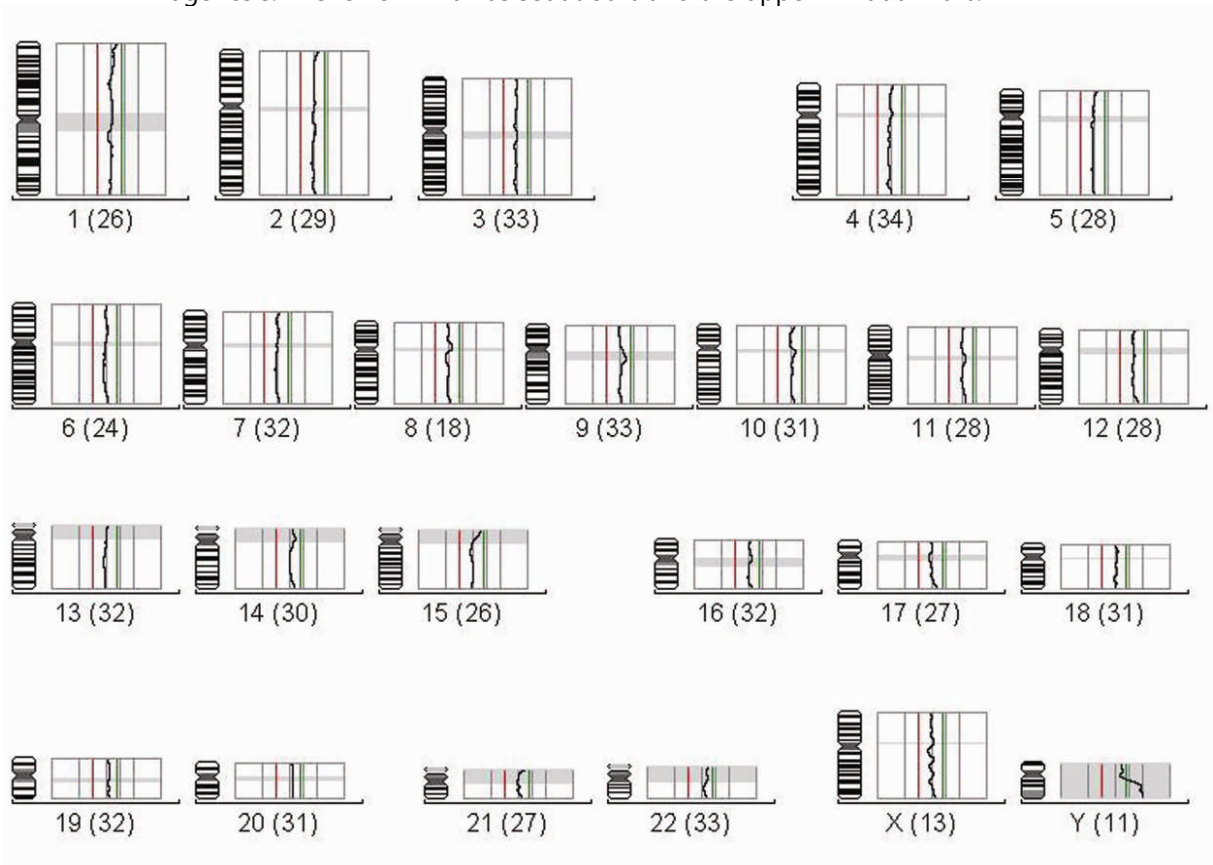
e *the father's feet.*

Chapter 7 Figure 3 (*see page 119*) Molecular model of MYCN

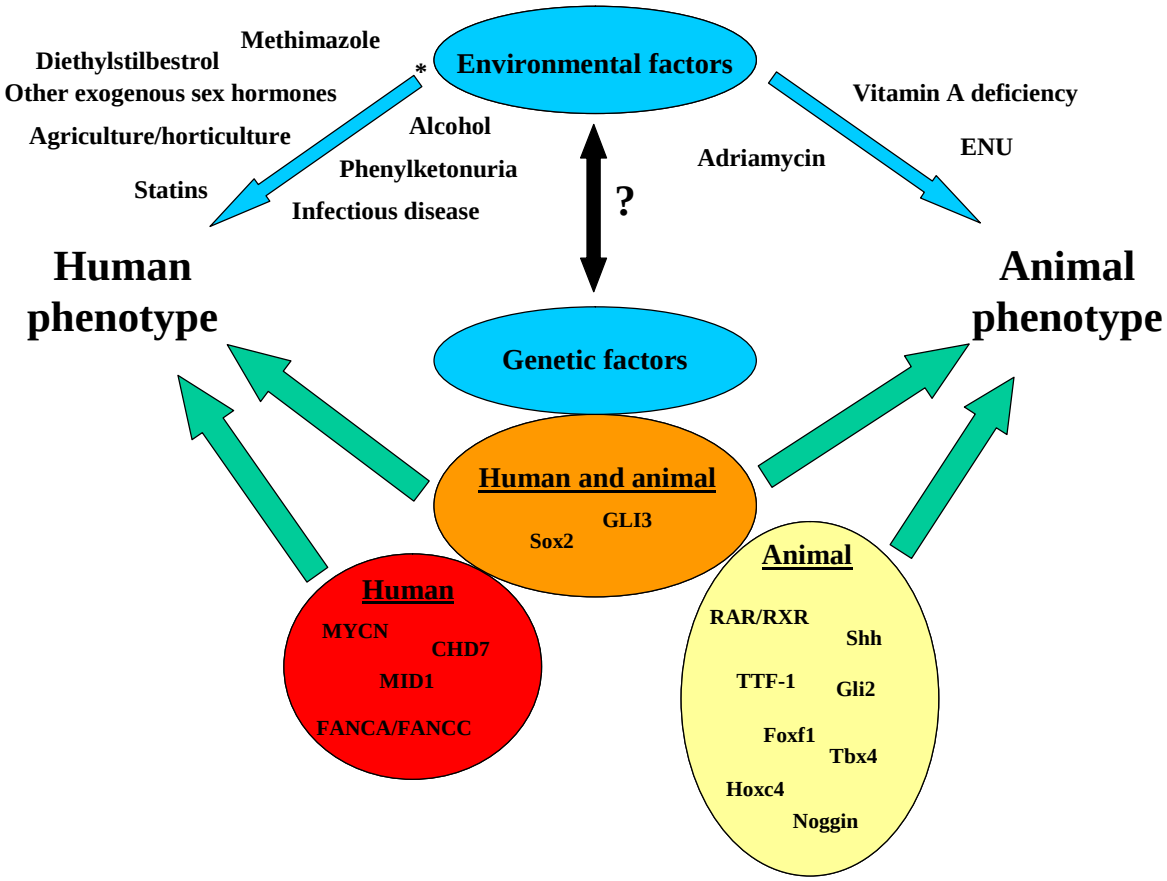


A *Interaction of the MYCN-MAX complex with DNA. The two MYCN alpha-helices are shown in dark blue, the loop in lighter blue. MAX is shown in gray and the DNA in orange. The location of P409 in the HLH loop is indicated in red.* ***B,C*** *Detailed view of the P409L amino acid residue substitution. MYCN is shown in blue. The HLH motif of MAX is shown in gray. Hydrogen bonds are indicated by yellow dotted lines.*

Chapter 10 Figure 1 (*see page 183*) Example of a normal CGH profile in a patient with tracheal agenesis. The lower limit was set at 80% and the upper limit at 120%.



Chapter 10 **Figure 2** (*see page 186*) Factors (possibly) involved in the aetiology of OA/TOF in animals and humans



*Proven in humans; Proven in humans and animals; Proven in animals; ? possible interactions as yet unknown; * factors with proven or suggested involvement. See text for details.*

Chapter 10 **Figure 3** (*see page 191*) Artist's impression by Jacob van der Goot of the aspects of research surrounding a child with a congenital anomaly