MSX1 in relation to clefting, hypodontia and hydrocephaly in humans

Marie-José van den Boogaard

Promotor: Prof. dr. D.Lindhout
Co-promotor: Dr. J.K. Ploos van Amstel

Concept & Design by Sabel Design (Michel van den Boogaard), Bilthoven
Lay out by Studio Voetnoot, Utrecht
Printed and bounded by Drukwerkconsultancy, Utrecht
ISBN 978-90-393-5903-7

Picture Cover: molar tooth bud mouse embryo (E12) – with thanks to D Sassoon and B Robert - Génétique Moléculaire de la Morphogenèse, Institut Pasteur, Paris, France.
Foto: Vincent Boon – www.vincentboon.nl

MSX1 in relation to clefting, hypodontia and hydrocephaly in humans

***MSX1* in relatie tot schisis, hypodontie en hydrocefalie bij de mens**
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. G.J. van der Zwaan, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op dinsdag 29 januari 2013 des middags te 4.15 uur.

door

Marie-José Henriette van den Boogaard
geboren op 2 augustus 1964 te Helmond

Promotor
Prof. dr. D. Lindhout

Co-promotor
Dr. J.K. Ploos van Amstel

Dit proefschrift werd mede mogelijk gemaakt met financiële steun van
de Nederlandse Vereniging voor Gnathologie en Prothetische Tandheelkunde (NVGPT).

Voor Paul, Bram, Freek en Evy

Manuscriptcommissie

Prof.dr. R. Koole

Prof.dr. M.S. Cune

Prof.dr. R.C.M. Hennekam

Prof.dr.ir. J.H.L.M. van Bokhoven

Paranimfen

M-L.E. van den Boogaard

P.A.Terhal

Contents

Chapter 1 **General introduction** **11**
Aims and Outline of the thesis

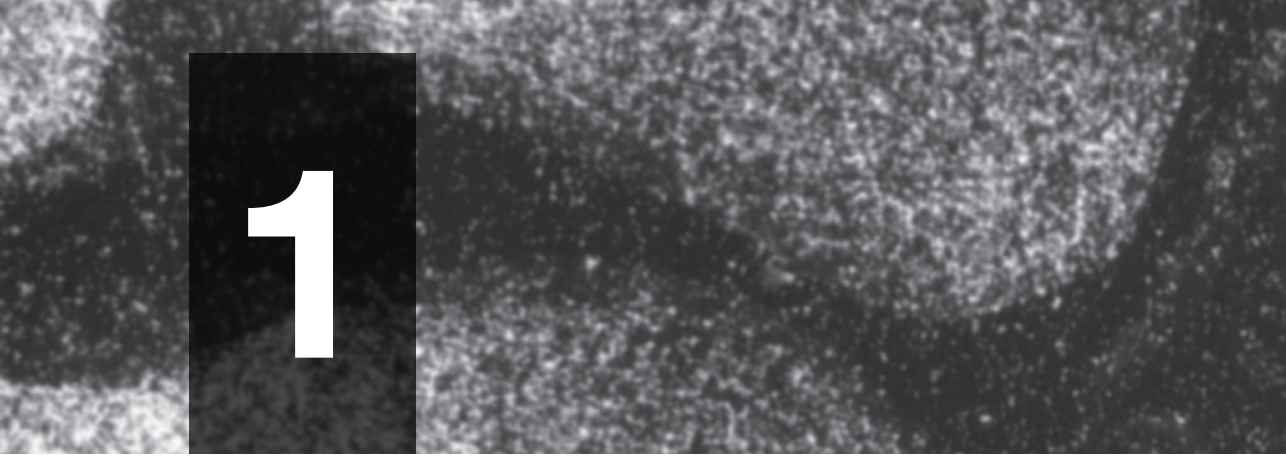
Chapter 2 **Orofacial Clefting and associated anomalies** **39**
A systematic review of associated structural and chromosomal defects in oral clefts
J Med Genet. 2012; 49: 490-8

Chapter 3 ***MSX1* causes clefting in humans** **75**
MSX1 mutation is associated with orofacial clefting and tooth agenesis in humans
Nat Genet. 2000; 24: 342-3

Chapter 4 **The role of *Msx1* in development** **83**
MSX1 and partial anodontia and the Witkop syndrome
Chapter 75: Inborn errors of development Inborn errors of development : the molecular basis of clinical disorders of morphogenesis / ed. by Charles J. Epstein, Robert P. Erickson, Anthony Wynshaw-Boris
2008 ISBN-13: 978-0-19-530691-0

Chapter 5 ***MSX1* and non-syndromic clefting** **125**
The *MSX1* allele 4 homozygous child exposed to smoking at periconception is most sensitive in developing nonsyndromic orofacial clefts
Hum Genet. 2008; 124: 525-34

Chapter 6	***WNT10A*, rather than *MSX1* is a major cause of non-syndromic hypodontia** Mutations in *WNT10A* are present in more than half of isolated hypodontia cases J Med Genet. 2012; 49: 327-331	149
Chapter 7	***MSX1* and tooth dimensions** Three – dimensional analysis of tooth dimensions in the *MSX1* – nonsense mutation Clin Oral Investig 2012 Aug 31. [Epub ahead of print]	177
Chapter 8	***MSX1* and hydrocephaly** Identification of a novel missense mutation in the *MSX1* homeodomain, in a patient with hydrocephaly Submitted Eur J Hum Genetics	199
Chapter 9	**General Discussion and Summary**	215
	Future Prospects	225
	Nederlandse Samenvatting (Summary in Dutch)	238
	Curriculum Vitae	243
	Dankwoord (Acknowledgements)	244
	List of Publications	250
	Addenda	254



1

Chapter 1

General introduction and aims and outline of the thesis

General introduction

A mother contacted our department because her son was born with an orofacial cleft, just like her brother, uncle and cousin.
Family history learned that the mother had an agenesis of more than 6 teeth. She remarked " there is nothing unusual about this, because almost all of her family members misses a few or a larger number of teeth."

Orofacial cleft and hypodontia

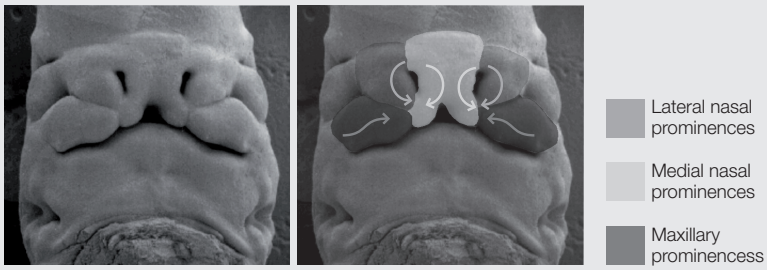
Orofacial clefting is a very common congenital abnormality. Clefts of the lip and palate are generally divided into two cagetories, cleft lip with or without cleft palate (CL±P) and isolated cleft palate (CP). [1] However, recent studies emphasized subdivision into three categories: cleft lip only (CL), cleft lip with cleft palate (CLP), and CP, because of differences concerning embryologic development, prevalence, risk factors, and associations with other congenital anomalies. [2-5]
For most people an orofacial cleft (CL and CLP account for 66% of the cleft cases) [3], is an immediately recognizable birth defect.
In the Netherlands approximately 300-350 children are born annually with a cleft lip and/or palate [6]. Often the newborn with cleft lip and/or palate is the first such a child in the family. However there is an increased risk for orofacial clefts to recur in families [7, 8]
The birth incidence of cleft lip with or without cleft palate (CL±P) and cleft palate (CP) show a striking variability that is dependent on geographic origins, ethnic groups but also on environmental exposures and socio-economic status. [1, 5, 9] The highest rates of birth prevalences are reported in certain (Asian and Native American) population and are as high as 1:500. African derived populations show the lowest birth incidences of 1:2500. In the European population an intermediate birth incidence of 1:1000 is observed. [9] The prevalence of orofacial clefting in the Netherlands is 1:600 live births. [6]
Cleft lip and/or palate are often associated with additional congenital anomalies. [5] An increasing number of studies demonstrate that in patients with CL±P or CP congenital tooth agenesis/hypodontia is significantly more frequent than in the general population. [10-19] The prevalence of hypodontia (excluding third molars or Wisdom teeth) in cases with orofacial clefting differs between populations and between types of clefts. The occurrence of hypodontia in cases with orofacial clefting ranges from approximately 5 % [10] to 77% [11] compared to 5,5 % in the general population. [20] Hypodontia can be attributed in part to the cleft itself or to the early surgical correction of the defects. However, hypodontia is also much more common outside the cleft region with reported frequencies

of approximately 30%. [12, 14, 17] A recent systematic review and meta-analyses showed a significant association between tooth agenesis and orofacial clefts. [21]
The increased rates of hypodontia in orofacial clefting suggest a related etiology. This might be based on the close anatomic relation and developmental timing, shared morphological processes, such as outgrowth and differentiation, and underlying molecular mechanisms during the embryonic development of the face and teeth.

Facial and tooth development: related processes

During the development of the face, the morphogenesis of the primary palate (the upper lip and alveolar process) and the secondary palate (the hard and soft palates including the uvula), and the teeth entail a complex series of events. [8, 9, 22-37]
The development of the face and the primary and secondary palates are the result of the narrowly coordinated formation, i.e. outgrowth, apoptosis and fusion of facial and palatal prominences/processes/shelves followed by their differentiation into musculature, bone and teeth (Figure 1.1 A/B and Figure 1.2). The fusion of the facial and palatal processes entails several steps. The facial and palatal processes consist of a mesenchymal core covered with ectoderm. Through outgrowth of the processes initial contact of the covering epithelial layers (ectoderm) of the opposite processes occur. After adhesion the intervening epithelium gradually disappear by the crucial process of apoptosis and epitheliomesenchymal transformation. [23, 28]
First, the primary palate is formed bilaterally by outgrowth and fusion of the median nasal process with the maxillary and lateral nasal processes, respectively, during the 4th -7th week after conception. Then, the secondary palate starts to develop by outgrowth of the palatal shelves, emerging from the maxillary processes, at 7th weeks after conception. After elevation of the vertically oriented palatal shelves the fusion of the shelves takes place with the primary palate, with each other and the nasal septum. The formation of the secondary palate is completed at the 12th week after conception. [9, 23-29] (see Figure 1.1 B)

Figure 1.1 A.



Human embryo, approximate human age 6 weeks

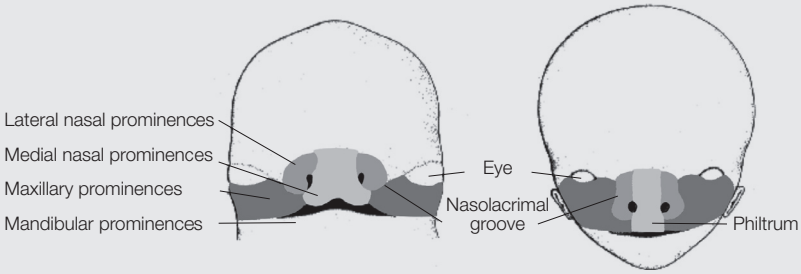


Figure 1.1 A. Embryological lip development | by Drs. Kathleen K. Sulik and Peter R. Bream Jr. with the assistance of Mr. Tim Poe and Ms. Kiran Bindra. https://syllabus.med.unc.edu/courseware/embryo_images/unit-hednk/hednk_htms/hednk031.htm and https://syllabus.med.unc.edu/courseware/embryo_images/unit-hednk/hednk_htms/hednk032.htm. Adapted with permission.

Figure 1.1 B.

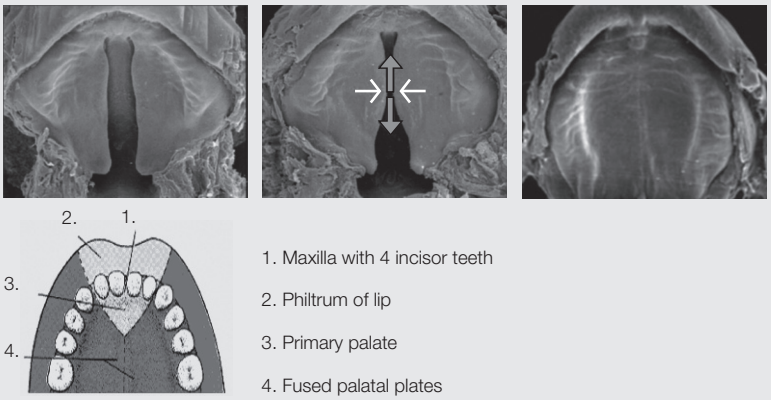


Figure 1.1 B. Embryologic palatal development | by Drs. Kathleen K. Sulik and Peter R. Bream Jr. with the assistance of Mr. Tim Poe and Ms. Kiran Bindra. Human embryo : approximate human age 9 – 10 weeks | https://syllabus.med.unc.edu/courseware/embryo_images/unit-hednk/hednk_htms/hednk039.htm and https://syllabus.med.unc.edu/courseware/embryo_images/unit-hednk/hednk_htms/hednk040.htm - Adapted with permission.

In the period the secondary palate develops, the fused medial nasal and maxillary processes of the primary palate form the presumptive upper lip, and alveolar process by their outgrowth in caudal direction, with the labial groove in between (see figure 1.1 A). [9, 23, 28] Then, within each maxillary process one maxillary bone center develops at the 7 th week and two bone centers will develop within each medial nasal process forming the premaxilla, at the 8 th and 11-12 th weeks after conception, respectively. [26] These bone centers, each premaxilla bears two incisors, grow out and form the maxilla including the alveolar process.

Tooth development is initiated at the ectoderm covering the presumptive alveolar process. As does facial development, tooth development proceeds through a well-characterized series of morphological and differentiation stages. [22, 30-35] The first sign is thickening of the oral ectoderm at approximately the 9th week of development, forming the dental placodes. The mesenchyme condenses under the thickened ectoderm and the epithelium starts to grow into in the underlying mesoderm. This epithelial invagination leads to the bud stage of the tooth development. The epithelium wraps around the condensing mesenchyme forming the cap stage and subsequently the bell stage. This process is thought to be controlled by the enamel knot, also referred to as the signaling

centre, formed at the tip of the late bud. [33] At the bell stage of development, cell differentiation occurs and the ameloblasts and odontoblasts are formed in adjacent layers at the site of interaction between the epithelium and mesenchyme. These layers produce the enamel and dentin of the fully formed tooth. [22, 30-34] (see also Figure 1.2). In the end, teeth are developed from the ectoderm as well as the mesoderm. The enamel originates from the ectoderm and the dentin, pulp and cement is formed from the mesoderm.

During different stages of tooth development, apoptosis is seen. High levels of apoptosis are noted within the enamel knot in the late cap and early bell stage, which lead to disappearance of the enamel knot and silencing of the signaling centre. [33]

At the bell stage, differentiating osteoblasts appear and surround the developing tooth, giving rise to the boney socket of the alveolus. In addition, the hard palate is formed by outgrowth of the maxillary bone centers into the palatal shelves and the development of the palatine bone centers at the 8th weeks after conception. During the bone differentiation of the primary and secondary palate, the musculature develops. [23, 26]

Thus, proliferation of the mesenchyme and apoptosis are the driving forces of facial morphogenesis. Failure of outgrowth or fusion of the facial and/or palatal processes/ shelves or failure of adequate differentiation after fusion leads to a spectrum of orofacial clefting. [23, 26]

Interruption of the fusionprocess of the facial processes/shelves will result in, for example, a complete cleft lip, alveolus and/or complete cleft palate. However, when fusion of the facial processes is completed, but caudal outgrowth of the lip is insufficient, an incomplete cleft lip will be present. [23] Insufficient outgrowth of bone centers of the premaxilla and maxilla causes submucous alveolar clefts. [23]

Figure 1.2

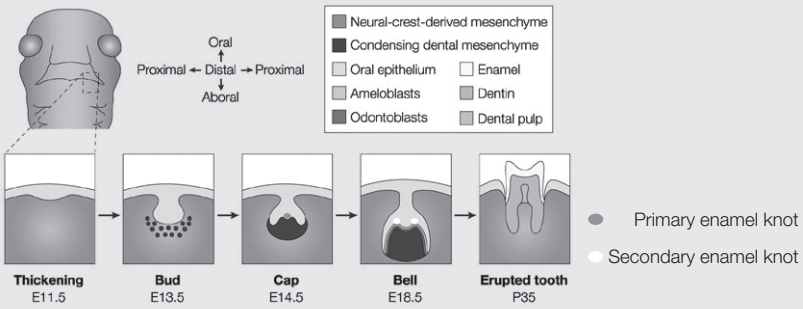


Figure 1.2 Tooth development in the mouse embryo | A schematic frontal view of an embryo head at embryonic day (E)11.5 is shown with a dashed box to indicate the site where the lower (mandibular) molars will form. Below, the stages of tooth development are laid out from the first signs of thickening at E11.5 to eruption of the tooth at around 5 weeks after birth. The tooth germ is formed from the oral epithelium and neural-crest-derived mesenchyme. At the bell stage of development, the ameloblasts and odontoblasts form in adjacent layers at the site of interaction between the epithelium and mesenchyme. These layers produce the enamel and dentin of the fully formed tooth. | By Tucker A, Sharpe P. The cutting-edge of mammalian development; how the embryo makes teeth. Nat Rev Genet. 2004 Jul;5(7):499-508. Adapted with permission by Nature Publishing Group.

Embryonic development of the face and teeth, and the above-described morphologic and differentiation processes involved, are the result of reciprocal interactions between the epithelium and mesenchyme. [22-25, 30-34] The proliferation and directed expansion of the facial mesenchyme depend on signals from the facial epithelia. At the same time, signals from the mesenchyme influence development of the facial ectoderm. [24] In tooth development, the epithelium induces odontogenic potential in the dental mesenchyme and guides the very early stages of tooth formation.
These reciprocal epithelial-mesenchymal interactions in facial and tooth development involve many intercellular signaling pathways, which have been conserved during evolution and are exploited throughout the embryonic stage in all kind of developmental processes. [24, 27, 28, 29-39]

These signaling pathways involve many genes and proteins expressed in the epithelium or mesenchyme, and lead to the expression or repression of specific sets of genes in the adjacent tissue. Binding of signaling molecules to specific cell-surface receptors initiate

a chain of molecular events, which can activate or inactivate specific transcription factors. Transcription factors are proteins that bind to regulatory regions in the DNA, resulting in the expression or repression of genes. These sets of genes include genes encoding structural proteins and proteins involved in diverse cell biological mechanisms such as proliferation, apoptosis and differentiation, which are of great importance in embryonic development including both facial and dental development. The complexity and diversity of morphogenetic events involved in the development of the face and teeth are reflected in the large number of genes involved. [24, 27, 28, 30-32]

Facial and tooth development: related signaling pathways

The members of the important signaling pathways that are involved in orofacial development belong mainly to four families: the Fibroblast Growth Factor (FGF) family, the Hedgehog (HH) family, the Transforming Growth Factor beta (TGF- β) family including the Bone Morphogenetic Proteins and the Activins and the Wingless (WNT) family.
Expression studies showed that several genes involved in these pathways, are expressed in the facial processes and in tooth initiation sites as well, supporting the role of these specific genes in both facial and dental development (e.g. FGFR1, SHH, TGF- β 1,2,3 and WNT3) [22, 36] Disturbance in these tightly balanced signaling pathways during facial embryogenesis and tooth development may result in orofacial clefting and/or dental defects. This disturbance might be due to mutations in genes encoding proteins comprising these signaling pathways, including ligands, receptors and transcription factors or environmental factors interfering with these pathways, or a combination of both.

To date, studies in mouse models and humans revealed several genes in these pathways causative for oral clefting in combination with tooth agenesis in humans. [22, 27, 28, 38]
To illustrate, we highlight a selection of the genes involved in the above-mentioned pathways.

FGFR1

Kallmann syndrome 2 (KAL2, OMIM 147950) is a rare autosomal dominant disorder characterized by hypogonadotropic hypogonadism with a defective sense of smell. These features due to a mutation in *FGFR1* are highly variable. In a few patients normal sense of smell has been reported. [40] Interestingly, cleft lip and/or palate may occur in as many as 25–30% of patients with Kallman syndrome 2, and also tooth agenesis is observed in patients with this syndrome. [41, 42] Sequencing of *FGF*-related genes in more

than 184 individuals with nonsyndromic CL±P revealed a nonsense *FGFR1* mutation in a father with isolated CL±P and a daughter with Kallmann syndrome. [42] An association study showed that isolated maxillary premolar agenesis appears to be associated with *FGFR1*. [43]

SHH

Mutations in various genes of the SHH (Sonic Hedgehog) signaling pathway might lead to a wide range of syndromes. [28, 44] Cell signaling of this pathway is initiated through binding of SHH to the transmembrane receptor Patched 1 (Ptc1). In the absence of SHH, PTC1 represses the activation of the transmembrane smoothened (Smo). By binding of SHH to PTC1, the basal repression of Smo is relieved and Smo activates Shh downstream components. [44] Mutations in *SHH* cause holoprosencephaly (HPE, OMIM 236100), with a striking variable expression ranging from severe brain anomalies with cyclopia to a subtle feature as mild cleft lip and palate, a single central incisor or close-set eyes. [44] Mutations in *PTCH1*, cause Basal Cell Nevus (Gorlin) syndrome (BCNS, OMIM 109400). [45] The main clinical features are multiple jaw cysts, palmar or plantar pits and basal cell carcinomas usually from the third decade and macrocephaly. [45] BCNS can be associated with CL±P and hypodontia. [45] An association study gave evidence that *PTCH1* contribute to the etiology of CL±P. [46] Sequence analysis in a large population of patients with isolated CL±P revealed *PTCH* may represent a rare cause. [47]

TGF-β

Mutations in a downstream target of TGF- β signaling, the transcription factor *IRF6*, can cause Van der Woude syndrome type 1 (VWS-1, OMIM 119300). [48, 49] Van der Woude syndrome (VWS) is characterized by pits and/or sinuses of the lower lip (lip pits), and CL±P or CP. VWS is the most common cleft syndrome and often features congenital tooth agenesis. [50] In a study on the correlations between microforms of VWS and CP, it was found that in CP individuals with lower lip sinuses the frequency of hypodontia was 77.8%. [51] A recent study revealed IRF6 plays a critical role in regulating tooth epithelial invagination in murine tooth development, which may explain this high frequency. [52] Association studies confirm IRF6 contributes to human tooth agenesis. [53,54] *IRF6* has also been consistently associated with CL±P in different populations. [55, 56] Interestingly, a certain genetic variant in a highly conserved *IRF6* enhancer was significantly over transmitted in families with CL. This variant was found to disrupt the binding site for the transcription factor AP-α. [57] Mutations in this gene *(TFAP2A*) result in branchio-oculo-facial syndrome (BOFS, OMIM 113620). The main features of BOFS are (Branchial)

cutaneous defect, Ocular anomalies and Facial clefts. Also, dental anomalies, including small teeth and oligodontia have been reported in cases with BOFS. [58-60]

Recent studies demonstrate cooperation between the transcription factors IRF6 and P63. [61, 62] Both genes are characterized by Gritli-Linde (2010) as brothers in arms against clefting. [63] It was shown that P63 and IRF6 operate within a regulatory loop to coordinate epithelial proliferation and differentiation during palate development. Mutations in *P63* are found in syndromes characterized by ectodermal dysplasia, abnormal limb abnormalities and cleft lip and/or palate (including Ectrodactyly, ectodermal dysplasia, and cleft lip/palate syndrome 3, (EEC3, OMIM 604292), ankyloblepharon-ectodermal defects-cleft lip/palate syndrome (AEC, OMIM 106260) and acro-dermato-ungual-lacrimal-tooth syndrome (ADULT, OMIM 103285). [64] Hypodontia is one of the recognizable features in these syndromes. [64, 65] This can be explained by the observation that P63 is required for the formation of the dental placodes. [65]

Recently, a functional link between FGF signaling and P63 in the expansion of epithelial progenitor cells during embryonic development has been identified. [66, 67] A P63 knock-in mouse model for AEC syndrome showed impairment of fibroblast growth factor (FGF) signaling resulting from reduced expression of *Fgfr2* and *Fgfr3*, direct P63 target genes. [66, 67] Interestingly, mice lacking the epithelial-specific Fgfr2b isoform, show epidermal hypoplasia associated with abnormal hair and tooth development and cleft palate. [68, 69] FGFR2 is expressed in the dental epithelium during the dental development and plays an important role in the dental development. [70] Mutations in *FGFGR2* might cause Lacrimo-Auriculo-Dento-Digital syndrome LADD syndrome (LADD, OMIM 149730). [71] LADD is a genetic heterogeneous disorder with lacrimal duct aplasia, hearing loss dental anomalies and digital anomalies. LADD syndrome shows a significant overlap with the above-mentioned ADULT syndrome, caused by mutations in *P63*. Recently, a study further supported the association of *FGFR2* and clefting. [42, 72] Interestingly, studies have demonstrated *Shh* is a downstreamtarget of Fgf10/Fgfr2b signaling. [69, 73] As above described SHH is an important signaling molecule involved in orofacial development.

WNT

The Wnt/ β-catenin signaling pathway regulates many aspects of development and disease. [74, 75, 76] Wnt signaling is essential as a regulator of the embryonic cell patterning, proliferation, differentiation, adhesion, survival and apoptosis. [76, 77, 78] Because these processes are crucial for facial and tooth development they imply a key role for Wnt/ β

-catenin signaling in facial tooth and development. [79,80] Indeed, several studies demonstrated involvement of *WNT* genes in the etiology of clefting and hypodontia in humans.
Mutations in *WNT3* have been associated with a rare recessive disorder tetra-amelia with orofacial clefting (OMIM 273395). [81] Mutations in *WNT7A* causes a range of limb malformations, including Fuhrmann syndrome (OMIM 228930) and the Al-Awadi/Raas-Rothschild/Schinzel phocomelia syndrome (AARRS). [82] Oral clefting was present in a few cases and teeth anomalies are noted in an unpublished case. [83, 84] Mutations in *WNT10A* underlie ectodermal dysplasia syndromes, including Odontoonychodermal dysplasia (OODD OMIM 257980) characterized by severe hypodontia, nail dystrophy, palmoplantar keratoderma, hyperhidrosis and hypotrichosis. [85] Furthermore, *WNT10A* mutations are present in isolated hypodontia. [86, 87]
Several association studies support the involvement of *WNT* genes in human orofacial clefting. [88, 89, 90, 91] The contribution of *WNT7A* [89] and *WNT10A* was suggested [88], the most consistent and strongest association was found for *WNT3*. [89, 90, 91]
Interestingly, mutations in *AXIN2*, a negative regulator of the WNT pathway causes familial tooth agenesis and increased risk of coloncancer. [92] Recent studies demonstrated association between *AXIN2* and orofacial clefting. [93, 94,95,96]

Another interesting finding is the identification of a conserved Pbx-wnt-P63-IRF6 regulatory model controlling face morphogenesis. [97] A generated mouse line, lacking Pbx genes, showed a perturbed Wnt signaling and exhibits orofacial clefting. This study revealed that Wnt controlled the expression of *p63,* which directly regulates *Irf6*. As above discussed both are well established cleft genes in humans.
In addition to these genes, it was demonstrated that also *Msx1 i*s a downstream target of the Wnt/ β-catenin signaling pathway during lip formation and fusion. [98,99]
Furthermore, MSX1 is involved in FGF [99, 100, 101], Shh [102, 103], TGF- β [101] and BMP signaling. [100, 101, 102, 103, 104, 106, 107]

MSX1

MSX1 is crucial for facial and tooth development. A loss of function mutation in *Msx1* leads to multiple craniofacial defects including clefting of the secondary palate, abnormalities of several facial bones and the malleus in the middle ear, and complete tooth agenesis. Some *Msx1* mutants develop hydrocephalus due to collapse of the cerebral aqueduct. [108, 109, 110,
In humans, a *MSX1* mutation was identified in a family with autosomal dominant selective tooth agenesis (OMIM 106600). [111] In this thesis we could identify *MSX1* causative for

monogenetic orofacial clefting in combination with tooth agenesis in the above presented family and established *MSX1* as a candidate gene for involvement in clefting in human. [112] Several association studies support the role of *MSX1* in the etiology of non-syndromic clefting in different populations. [113-126]

To date, several *MSX1* mutations have been reported in patients with hypodontia or cleft. (Table 1.1)

Tabel 1.1 Published *MSX1* Mutations

Mutation (cDNA)#	Mutation (protein)#	Previously known as
c.065G>A	p.Gly22Asp	p.Gly16Asp
c.119C>G	p.Ala40Gly	p.Ala34Gly
c.127A>C	p.Met43Leu	p.Met37Leu
c.200T>A	p.Met67Lys	p.Met61Lys
c.251A>T	p.Glu84Val	p.Glu78Val
c.290G>A	p.Gly97Asp	p.Gly91Asp
c.311G>A	p.Gly104Glu	p.Gly98Glu
c.332C>A	p.Ser111*	p.Ser105Stop
c.359T>G	p.Val120Gly	p.Val114Gly
c.365G>A	p.Gly122Glu	p.Gly116Glu
c.458C>A	p.Pro153Gln	p.Pro147Gln
c.471G>T	p.Arg157Ser	p.Arg151Ser
c.526C>T	p.Arg176Trp	p.Arg170Trp
c.577C>T	p.Gln193*	p.Gln187Stop
c.581C>T	p.Lys194Arg	p.Lys188Arg
c.583C>T	p.Gln195*	p.Gln189Stop
c.599C>T	p.Ala200Val	p.Ala194Val
c.605G>C	p.Arg202Pro	p.Arg196Pro
c.605G>A	p.Arg202His	p.Arg196His
c.623C>A	p.Ser208*	p.Ser202Stop
c.668G>T	p.Arg223Leu	p.Arg217Leu
c.673G>A	p.Ala225Thr	p.Ala219Thr
c.680C>A	p.Ala227Glu	p.Ala221Glu
c.689T>C	p.Leu230Pro	p.Leu224Pro
c.817G>T	p.Gly273Cys	p.Gly267Cys
c.818G>C	p.Gly273Ala	p.Gly267Ala
c.850C>T	p.Pro284Ser	p.Pro278Ser
c.470-2A>G	IVS1 -2 A>G	p.?
c.80dupG	p.Gly28Argfs*147	p.G22RfsX168
c.644dupA	p.Gln216Glyfs*124	p.Gln216GlnfsX125
c.(?_-235)-(*793_?)del	(del exon1-2) p.O?	(del exon 1-2)

Nomenclature of the mutations is according to HGVS with the reference sequence NM_002448.3

Table 1.1

Phenotype	Reference	Additional information
Cleft lip and palate	127	
Cleft lip with or without cleft palate	120	
	121	
	127	
	128	Also in controls – 128, 129
	129	Not significantly associated- 129
Cleft lip and palate	129	Nonpenetrant in father- 129
Tooth agenesis	130	
Cleft lip with cleft palate	128	
Cleft palate	128	
Cleft lip and palate	120	Nonpenetrant in father
Tooth agenesis and orofacial clefting	112	Reduced penetrance for cleft -112
Cleft lip	128	
Cleft lip and palate	128	
Cleft lip and palate	120	
	121	Also in controls – no association - 121
	131	Non-penetrant in father/grandmother- 131
	132	Association - 132
		Poly Phen - benign
Cleft lip and palate	128	
Tooth agenesis	133	
Tooth agenesis	134	
Hydrocephaly	135	
Tooth agenesis and cleft lip	136	No cleft in 2 children - 135
Tooth agenesis	137	Non-penetrant in 2 family members -136
Tooth agenesis	111	
Tooth agenesis and orofacial clefting	138	
Witkop syndrome	139	
Tooth agenesis	140	
Tooth agenesis	141	
Tooth agenesis	142	
Tooth agenesis	143	
Cleft lip and cleft palate	121	
Incomplete cleft lip	129	Nonpenetrant in father -129
Cleft lip	121	
Tooth agenesis	144	
Tooth agenesis	145	
Tooth agenesis	133	
Oligodontia in Wolf-Hirschhorn syndrome	146	

To give more insight in the molecular pathogenesis of clefting and hypodontia, in this introduction a few molecular pathways and genes involved in the etiology of these congenital anomalies are discussed. It is demonstrated that different pathways – can be involved in both non-syndromic and syndromic clefting and/or hypodontia. Several studies also provided evidence of interaction between signaling pathways and overlap between the phenotypic spectrum of syndromes associated with clefting and hypodontia. *MSX1* is one of the numerous genes, playing a significant role in biological and molecular mechanisms during the embryonic development of the face and teeth and involved in the etiology of clefting and hypodontia.
The research presented in this thesis focuses on *MSX1* in relation to clefting, hypodontia and hydrocephaly in humans.

Aims of this thesis

The general aim of this thesis is expanding the knowledge on the genetic basis of clefting, hypodontia and hydrocephaly to improve diagnostics and genetic counseling of patients and their family members.

Orofacial development and tooth development are closely connected in timing, morphological processes, cell biological functions and molecular mechanisms. A growing number of genes is identified that are involved in the etiology of both clefting and hypodontia.

MSX1 was appreciated as an interesting candidate gene for involvement in both cleft palate and selective tooth agenesis in human and a possible monogenetic cause of orofacial clefting in combination with hypodontia. This was based on the role of *Msx1* in both facial and dental development in mice, the observations made in *Msx1*-deficient mice and the results of association studies in human.

In 2000 we described the identification of *MSX1* as a causative gene for monogenetic orofacial clefting in combination with hypodontia in a Dutch family. This specifically triggered further study on *MSX1* in relation to orofacial clefting, hypodontia and hydrocephaly.

In this thesis the aims are to gain insight into

1. The prevalence in general of associated anomalies and chromosomal defects in orofacial clefting.
2. The *MSX1* gene as a candidate for monogenetic clefting in combination with hypodontia in humans.
3. The role of *MSX1* during embryonic development.
4. The contribution of *MSX1* in non-syndromic orofacial clefting and hypodontia in the Dutch population.
5. The frequency of mutations in *MSX1* and other candidate genes as cause of non-syndromic hypodontia.
6. The dental features that associate in the *MSX1* cleft family.
7. The contribution of *MSX1* in the etiology of congenitial hydrocephaly due to aqueduct stenosis in human.

Outline of this thesis

In **chapter 2** the prevalence of associated anomalies and chromosomal defects for different cleft categories is presented in a systematic review. **Chapter 3** reports on the identification of a family with orofacial clefting in addition to dental agenesis with a *MSX1* nonsense mutation. This is the first report presenting *MSX1* as monogenetic cause for clefting in combination with hypodontia in humans. It confirms *MSX1* as a candidate gene for clefting in humans. **Chapter 4** gives insight in the role *of MSX1* during embryonic development and discusses the developmental pathogenesis of *MSX1* mutations.
Chapter 5 describes a Dutch case-control triad (mother, father, and child) study on the interactions between *MSX1* and the periconceptional lifestyle e.g. smoking of parents in relation to the risk of orofacial clefts in their offspring. A specific allelic variant of *MSX1* is identified that significantly increases orafacial clefting when interacted with smoking. **Chapter 6** describes the frequency of *MSX1* mutations and mutations in additional candidate genes e.g. *PAX9, IRF6, AXIN2* and *WNT10A* in Dutch patients with non-syndromic hypodontia. Mutations in the *WNT10A* gene are identified as the most frequent cause.
In **Chapter 7** the differences in the tooth crown morphology between patients with a *MSX1* nonsense mutation and non-affected controls, measured with a *3D* technique, are studied. Quantification of tooth crown shapes might contribute to early identification of *MSX1* related hypodontia. **Chapter 8** focuses on hydrocephaly as a clinical feature possibly associated with *MSX1* mutations.
In the final **chapter 9** the importance of insight in the molecular pathways involved in human development in the identification of causative genes for specific phenotypes of dysmorphology is discussed.

Nomenclature of the mutations in this thesis is according to HGVS.
For tooth numbering see page 269.

References

1. Mossey PA, Little J, Munger RG, Dixon MJ, Shaw WC. Cleft lip and palate. Lancet. 2009;374:1773-85.
2. Rahimov F, Jugessur A, Murray JC. Genetics of nonsyndromic orofacial clefts. Cleft Palate Craniofac J. 2012;49:73-91.
3. Luijsterburg AJ, Vermeij-Keers C. Ten years recording common oral clefts with a new descriptive system. Cleft Palate Craniofac J. 2011;48:173-82.
4. Vermeij-keers C, Rozendaal AM, van den Boogaard M-JH , Mink van der Molen AB, Pajkrt E New ultrasound classification of oral and craniofacial clefts based on embryology. Manuscript in preparation.
5. Maarse W, Rozendaal AM, Pajkrt E, Vermeij-Keers C, Mink van der Molen AB, van den Boogaard MJ. A systematic review of associated structural and chromosomal defects in oral clefts: when is prenatal genetic analysis indicated? J Med Genet. 2012;49:490-8.
6. Rozendaal AM, Mohangoo AD, Ongkosuwito EM, Buitendijk SE, Bakker MK, Vermeij-Keers C. Regional variation in prevalence of oral cleft live births in the Netherlands 1997-2007: Time-trend analysis of data from three Dutch registries. Am J Med Genet A 2011 nov 21 [Epub ahead of print]
7. Sivertsen A, Wilcox AJ, Skjaerven R, Vindenes HA, Abyholm F, Harville E, Lie RT. Familial risk of oral clefts by morphological type and severity: population based cohort study of first degree relatives. BMJ. 2008;336:432-4.
8. Grosen D, Chevrier C, Skytthe A, Bille C, Mølsted K, Sivertsen A, Murray JC, Christensen K. A cohort study of recurrence patterns among more than 54,000 relatives of oral cleft cases in Denmark: support for the multifactorial threshold model of inheritance. J Med Genet. 2010;47:162-8.
9. Dixon MJ, Marazita ML, Beaty TH, Murray JC. Cleft lip and palate: understanding genetic and environmental influences. Nat Rev Genet. 2011;12:167-78.
10. Ranta R. A review of tooth formation in children with cleft lip/palate.

    Am J Orthod Dentofacial Orthop. 1986; 90:11-8. Shapira Y, Lubit E, Kuftinec MM. Hypodontia in children with various types of clefts. Angle Orthod. 2000;70:16-21.
11. Shapira Y, Lubit E, Kuftinec MM. Hypodontia in children with various types of clefts. Angle Orthod. 2000;70:16-21.
12. Eerens K, Vlietinck R, Heidbuchel K, Van Olmen A, Derom C, Willems G, et al. Hypodontia and tooth formation in groups of children with cleft, siblings without cleft, and nonrelated controls. Cleft Palate Craniofac J 2001;38:374-8.
13. Heliövaara A, Ranta R, Rautio J. Dental abnormalities in permanent dentition in children with submucous cleft palate. Acta Odontol Scand. 2004;62:129-31.
14. Letra A, Menezes R, Granjeiro JM, Vieira AR. Defining subphenotypes for oral clefts based on dental development. J Dent Res. 2007;86:986-91.
15. Menezes R, Vieira AR. Dental anomalies as part of the cleft spectrum. Cleft Palate Craniofac J 2008; 45:414–419.
16. Al Jamal GA, Hazza'a AM, Rawashdeh MA. Prevalence of dental anomalies in a population of cleft lip and palate patients. Cleft Palate Craniofac J. 2010;47:413-20.
17. Bartzela TN, Carels CE, Bronkhorst EM, Rønning E, Rizell S, Kuijpers-Jagtman AM. Tooth agenesis patterns in bilateral cleft lip and palate. Eur J Oral Sci. 2010;118:47-52.

18. Tereza GP, Carrara CF, Costa B. Tooth abnormalities of number and position in the permanent dentition of patients with complete bilateral cleft lip and palate. Cleft Palate Craniofac J. 2010;47:247-52.
19. Pegelow M, Alqadi N, Linder-Aronson Karsten A. The prevalence of various dental characteristics in the primary and mixed dentition in patients born with non-syndromic unilateral cleft lip with or without cleft palate. Eur J Orthod. 2012;34:561-70.
20. Polder BJ, Van't Hof MA, Van der Linden FP, Kuijpers-Jagtman AM. A meta-analysis of the prevalence of dental agenesis of permanent teeth. Community Dent Oral Epidemiol 2004; 32: 217-226.
21. Tannure PN, Oliveira CA, Maia LC, Vieira AR, Granjeiro JM, de Castro Costa M. Prevalence of Dental Anomalies in Nonsyndromic Individuals With Cleft Lip and Palate: A Systematic Review and Meta-analysis. Cleft Palate Craniofac J. 2012;49:194-200.
22. Kouskoura T, Fragou N, Alexiou M, John N, Sommer L, Graf D, Katsaros C, Mitsiadis TA. The genetic basis of craniofacial and dental abnormalities. Schweiz Monatsschr Zahnmed. 2011;121:636-46.
23. Krapels IP, Vermeij-Keers C, Muller M, De Klein A, Steegers-Theunissen RP. Nutrition and genes in the development of orofacial clefting. Nutr Rev 2006;64:280–8.
24. Jiang R, Bush JO, Lidral AC. Development of the upper lip: morphogenetic and molecular mechanisms. Dev Dyn. 2006;235:1152-66.
25. Gritli-Linde A. Molecular control of secondary palate development. Dev Biol. 2007;301:309-26.
26. Vermeij-Keers C. Craniofacial embryology and morphogenesis: normal and abnormal. In: Stricker M, Van der Meulen JC, Raphael B, Mazzola R, Tolhurst DE, Murray JE, eds. Craniofacial Malformations. Edinburgh, Scotland: Churchill Livingstone, 1990: 27–60.
27. Gritli-Linde A. The etiopathogenesis of cleft lip and cleft palate: usefulness and caveats of mouse models.Curr Top Dev Biol. 2008;84:37-138.
28. Jugessur A, Farlie PG, Kilpatrick N. The genetics of isolated orofacial clefts: from genotypes to subphenotypes. Oral Dis. 2009;15:437-53.
29. Greene RM, Pisano MM. Palate morphogenesis: Current understanding and future directions. Birth Defects Res C Embryo Today. 2010;90:133-54.
30. Thesleff I. The genetic basis of tooth development and dental defects. Am J Med Genet A. 2006;140:2530-5.
31. Thesleff I, Tummers M. Tooth organogenesis and regeneration. StemBook [Internet]. Cambridge (MA): Harvard Stem Cell Institute; 2008-2009 Jan 31.
32. De Coster PJ, Marks LA, Martens LC, Huysseune A. Dental agenesis: genetic and clinical perspectives. J Oral Pathol Med. 2009;38:1-17.
33. Tucker A, Sharpe P. The cutting-edge of mammalian development; how the embryo makes teeth. Nat Rev Genet. 2004;5:499-508.
34. Catón J, Tucker AS. Current knowledge of tooth development: patterning and mineralization of the murine dentition. J Anat. 2009;214:502-15.
35. Nieminen P. Genetic basis of tooth agenesis. J Exp Zool B Mol Dev Evol. 2009;312B:320-42.
36. Tummers M, Thesleff I. The importance of signal pathway modulation in all aspects of tooth development. J Exp Zool B Mol Dev Evol. 2009;312B:309-19.
37. Vukojevic K, Kero D, Novakovic J, Kalibovic Govorko D, Saraga-Babic M. Cell proliferation and apoptosis in the fusion of human primary and secondary palates. Eur J Oral Sci. 2012;120:283-91.

38. Cobourne MT, Sharpe PT. Making up the numbers: The molecular control of mammalian dental formula. Semin Cell Dev Biol. 2010;21:314-24.

39. Matalova E, Fleischmannova J, Sharpe PT, Tucker AS. Tooth agenesis: from molecular genetics to molecular dentistry.J Dent Res. 2008;87:617-23.

40. Dodé C, Hardelin JP. Kallmann syndrome. Eur J Hum Genet. 2009;17:139-46.

41. Bailleul-Forestier I, Gros C, Zenaty D, Bennaceur S, Leger J, de Roux N. Dental agenesis in Kallmann syndrome individuals with FGFR1 mutations. Int J Paediatr Dent. 2010;20:305-12.

42. Riley BM, Mansilla MA, Ma J, Daack-Hirsch S, Maher BS, Raffensperger LM, Russo ET, Vieira AR, Dodé C, Mohammadi M, Marazita ML, Murray JC. Impaired FGF signaling contributes to cleft lip and palate. Proc Natl Acad Sci U S A. 2007;104:4512-7.

43. Vieira AR, Modesto A, Meira R, Barbosa AR, Lidral AC, Murray JC. Interferon regulatory factor 6 (IRF6) and fibroblast growth factor receptor 1 (FGFR1) contribute to human tooth agenesis. Am J Med Genet A. 2007;143:538-45.

44. Iannaccone P, Holmgren R, Lamm M, Ahlgren S, Lakiza O, Yoon JW, Walterhouse D. The Sonic Hedgehog Signaling Pathway. In: Epstein CJ, Erickson RP, Wynshaw-Boris A, editors. 2008. Inborn Errors of Development, second edition. Oxford: Oxford University Press 263-279.

45. Lo Muzio L. Nevoid basal cell carcinoma syndrome (Gorlin syndrome). Orphanet J Rare Dis. 2008;3:32.

46. Carter TC, Molloy AM, Pangilinan F, Troendle JF, Kirke PN, Conley MR, Orr DJ, Earley M, McKiernan E, Lynn EC, Doyle A, Scott JM, Brody LC, Mills JL. Testing reported associations of genetic risk factors for oral clefts in a large Irish study population. Birth Defects Res A Clin Mol Teratol. 2010;88:84-93.

47. Mansilla MA, Cooper ME, Goldstein T, Castilla EE, Lopez Camelo JS, Marazita ML, Murray JC. Contributions of PTCH gene variants to isolated cleft lip and palate. Cleft Palate Craniofac J. 2006;43:21-9.

48. Knight AS, Schutte BC, Jiang R, Dixon MJ. Developmental expression analysis of the mouse and chick orthologues of IRF6: the gene mutated in Van der Woude syndrome. Dev Dyn. 2006;235:1441-7.

49. Xu X, Han J, Ito Y, Bringas P Jr, Urata MM, Chai Y. Cell autonomous requirement for Tgfbr2 in the disappearance of medial edge epithelium during palatal fusion. Dev Biol. 2006;297:238-48.

50. Oberoi S, Vargervik K. Hypoplasia and hypodontia in Van der Woude syndrome. Cleft Palate Craniofac J. 2005;42:459-66.

51. Ranta R, Rintala AE. Correlations between microforms of the Van der Woude syndrome and cleft palate. Cleft Palate J. 1983;20:158-62.

52. Blackburn J, Ohazama A, Kawasaki K, Otsuka-Tanaka Y, Liu B, Honda K, Rountree RB, Hu Y, Kawasaki M, Birchmeier W, Schmidt-Ullrich R, Kinoshita A, Schutte BC, Hammond NL, Dixon MJ, Sharpe PT. The role of Irf6 in tooth epithelial invagination. Dev Biol. 2012;365:61-70.

53. Vieira AR, Modesto A, Meira R, Barbosa AR, Lidral AC, Murray JC. Interferon regulatory factor 6 (IRF6) and fibroblast growth factor receptor 1 (FGFR1) contribute to human tooth agenesis. Am J Med Genet A. 2007;143:538-45.

54. Vieira AR, Seymen F, Patir A, Menezes R. Evidence of linkage disequilibrium between polymorphisms at the IRF6 locus and isolate tooth agenesis, in a Turkish population. Arch Oral Biol. 2008;53:780-4.

55. Jugessur A, Rahimov F, Lie RT, Wilcox AJ, Gjessing HK, Nilsen RM, Nguyen TT, Murray JC. Genetic variants in IRF6 and the risk of facial clefts: single-marker and haplotype-based analyses in a population-based case-control study of facial clefts in Norway. Genet Epidemiol. 2008;32:413-24.

56. Vieira AR, McHenry TG, Daack-Hirsch S, Murray JC, Marazita ML. Candidate gene/loci studies in cleft lip/palate and dental anomalies finds novel susceptibility genes for clefts. Genet Med. 2008;10:668-74.

57. Rahimov F, Marazita ML, Visel A, Cooper ME, Hitchler MJ, Rubini M, Domann FE, Govil M, Christensen K, Bille C, Melbye M, Jugessur A, Lie RT, Wilcox AJ, Fitzpatrick DR, Green ED, Mossey PA, Little J, Steegers-Theunissen RP, Pennacchio LA, Schutte BC, Murray JC. Disruption of an AP-2alpha binding site in an IRF6 enhancer is associated with cleft lip. Nat Genet. 2008;40:1341-7.

58. Milunsky JM, Maher TA, Zhao G, Roberts AE, Stalker HJ, Zori RT, Burch MN, Clemens M, Mulliken JB, Smith R, Lin AE. TFAP2A mutations result in branchio-oculo-facial syndrome. Am J Hum Genet. 2008;82:1171-7. Erratum in: Am J Hum Genet. 2009;84:301.

59. Milunsky JM, Maher TM, Zhao G, Wang Z, Mulliken JB, Chitayat D, Clemens M, Stalker HJ, Bauer M, Burch M, Chénier S, Cunningham ML, Drack AV, Janssens S, Karlea A, Klatt R, Kini U, Klein O, Lachmeijer AM, Megarbane A, Mendelsohn NJ, Meschino WS, Mortier GR, Parkash S, Ray CR, Roberts A, Roberts A, Reardon W, Schnur RE, Smith R, Splitt M, Tezcan K, Whiteford ML, Wong DA, Zori R, Lin AE. Genotype-phenotype analysis of the branchio-oculo-facial syndrome. Am J Med Genet A. 2011;155A:22-32.

60. Galliani E, Burglen L, Kadlub N, Just W, Sznajer Y, Billette de Villemeur T, Soupre V, Picard A, Vazquez MP. Craniofacial Phenotype in the Branchio-Oculo-Facial Syndrome: Four Case Reports. Cleft Palate Craniofac J. 2012;49:357-64.

61. Thomason HA, Zhou H, Kouwenhoven EN, Dotto GP, Restivo G, Nguyen BC, Little H, Dixon MJ, van Bokhoven H, Dixon J Cooperation between the transcription factors p63 and IRF6 is essential to prevent cleft palate in mice. J Clin Invest. 2010;120:1561-9.

62. Moretti F, Marinari B, Lo Iacono N, Botti E, Giunta A, Spallone G, Garaffo G, Vernersson-Lindahl E, Merlo G, Mills AA, Ballarò C, Alemà S, Chimenti S, Guerrini L, Costanzo A.A regulatory feedback loop involving p63 and IRF6 links the pathogenesis of 2 genetically different human ectodermal dysplasias.J Clin Invest. 2010;120:1570-7.

63. Gritli-Linde A. p63 and IRF6: brothers in arms against cleft palate. J Clin Invest. 2010;120:1386-9.

64. Rinne T, Brunner HG, van Bokhoven H. p63-associated disorders. Cell Cycle. 2007;6:262-8.

65. Laurikkala J, Mikkola ML, James M, Tummers M, Mills AA, Thesleff I. p63 regulates multiple signalling pathways required for ectodermal organogenesis and differentiation. Development. 2006;133:1553-63.

66. Ferone G, Thomason HA, Antonini D, De Rosa L, Hu B, Gemei M, Zhou H, Ambrosio R, Rice DP, Acampora D, van Bokhoven H, Del Vecchio L, Koster MI, Tadini G, Spencer-Dene B, Dixon M, Dixon J, Missero C. Mutant p63 causes defective expansion of ectodermal progenitor cells and impaired FGF signalling in AEC syndrome. EMBO Mol Med. 2012;4:192-205.

67. Dotto GP. p63 and FGFR: when development meets proliferation.EMBO Mol Med. 2012;4:165-7.

68. De Moerlooze L, Spencer-Dene B, Revest JM, Hajihosseini M, Rosewell I, Dickson C. An important role for the IIIb isoform of fibroblast growth factor receptor 2 (FGFR2) in mesenchymal-epithelial signalling during mouse organogenesis. Development. 2000;127:483-92.

69. Rice R, Spencer-Dene B, Connor EC, Gritli-Linde A, McMahon AP, Dickson C, Thesleff I, Rice DP. Disruption of Fgf10/Fgfr2b-coordinated epithelial-mesenchymal interactions causes cleft palate. J Clin Invest. 2004;113:1692-700.

70. Galluccio G, Castellano M, La Monaca C. Genetic basis of non-syndromic anomalies of human tooth number. Arch Oral Biol. 2012;57:918-30.

71. Rohmann E, Brunner HG, Kayserili H, Uyguner O, Nürnberg G, Lew ED, Dobbie A, Eswarakumar VP, Uzumcu A, Ulubil-Emeroglu M, Leroy JG, Li Y, Becker C, Lehnerdt K, Cremers CW, Yüksel-Apak M, Nürnberg P, Kubisch C, Schlessinger J, van Bokhoven H, Wollnik B. Mutations in different components of FGF signaling in LADD syndrome. Nat Genet. 2006;38:414-7.

72. Wang H, Zhang T, Wu T, Hetmanski JB, Ruczinski I, Schwender H, Murray T, Fallin MD, Redett RJ, Raymond GV, Jin SC, Wu-Chou YH, Chen PK, Yeow V, Chong SS, Cheah FS, Jee SH, Jabs EW, Liang KY, Scott A, Beaty TH. The FGF&FGFR Gene Family and Risk of Cleft Lip with/without Cleft Palate. Cleft Palate Craniofac J. 2011 Nov 10. [Epub ahead of print]

73. Revest JM, Spencer-Dene B, Kerr K, De Moerlooze L, Rosewell I, Dickson C. Fibroblast growth factor receptor 2-IIIb acts upstream of Shh and Fgf4 and is required for limb bud maintenance but not for the induction of Fgf8, Fgf10, Msx1, or Bmp4. Dev Biol. 2001;231:47-62.

74. Clevers H, Nusse R. Wnt/α-catenin signaling and disease. Cell. 2012 Jun 8;149(6):1192-205.

75. Clevers H. Wnt/beta-catenin signaling in development and disease. Cell. 2006;127:469-80.

76. Nusse R. Wnt signaling. Cold Spring Harb Perspect Biol. 2012 May 1;4(5). Review

77. Pe´cina-Slaus N. Wnt signal transduction pathway and apoptosis: a review. Cancer Cell Int. 2010;10:22.

78. Paiva KB, Silva-Valenzuela MG, Massironi SM, Ko GM, Siqueira FM, Nunes FD. Differential Shh, Bmp and Wnt gene expressions during craniofacial development in mice. Acta Histochem. 2010;112:508-17.

79. He F, Chen Y. Wnt signaling in lip and palate development. Front Oral Biol. 2012;16:81-90.

80. Liu F, Millar SE. Wnt/beta-catenin signaling in oral tissue development and disease. J Dent Res. 2010;89:318-30.

81. Niemann S, Zhao C, Pascu F, Stahl U, Aulepp U, Niswander L, Weber JL, Muller U. Homozygous WNT3 mutation causes tetra-amelia in a large consanguineous family. Am J Hum Genet. 2004;74:558–63.

82. Woods CG, Stricker S, Seemann P, Stern R, Cox J, Sherridan E, Roberts E, Springell K, Scott S, Karbani G, Sharif SM, Toomes C, Bond J, Kumar D, Al-Gazali L, Mundlos S. Mutations in WNT7A cause a range of limb malformations, including Fuhrmann syndrome and Al-Awadi/Raas-Rothschild/Schinzel phocomelia syndrome. Am J Hum Genet. 2006;79:402-8.

83. Alp E, Alp H, Atabek ME, Pirgon Ö. Al-Awadi/Raas-Rothschild syndrome in a newborn with additional anomalies. J Clin Res Pediatr Endocrinol. 2010;2:49-51.

84. Lonardo F, Sabba G, Luquetti DV, Monica MD, Scarano G. Al-Awadi/Raas-Rothschild syndrome: two new cases and review. Am J Med Genet A. 2007;143A:3169-74.

85. Adaimy L, Chouery E, Megarbane H, Mroueh S, Delague V, Nicolas E, Belguith H, de Mazancourt P, Megarbane A. Mutation in WNT10A is associated with an autosomal recessive ectodermal dysplasia: the odonto-onycho-dermal dysplasia. Am J Hum Genet. 2007;81:821-8.

86. Kantaputra P, Sripathomsawat W. WNT10A and isolated hypodontia. Am J Med Genet A. 2011;155A:1119-22.

87. van den Boogaard MJ, Créton M, Bronkhorst Y, van der Hout A, Hennekam E, Lindhout D, Cune M, Ploos van Amstel HK. Mutations in WNT10A are present in more than half of isolated hypodontia cases. J Med Genet. 2012;49:327-31.

88. Beaty TH, Hetmanski JB, Fallin MD, Park JW, Sull JW, McIntosh I, Liang KY, Vanderkolk CA, Redett RJ, Boyadjiev SA, Jabs EW, Chong SS, Cheah FS, Wu-Chou YH, Chen PK, Chiu YF, Yeow V, Ng IS, Cheng J, Huang S, Ye X, Wang H, Ingersoll R, Scott AF. Analysis of candidate genes on chromosome 2 in oral cleft case-parent trios from three populations. Hum Genet. 2006;120:501-18.

89. Chiquet BT, Blanton SH, Burt A, Ma D, Stal S, Mulliken JB, Hecht JT. Variation in WNT genes is associated with non-syndromic cleft lip with or without cleft palate. Hum Mol Genet. 2008;17:2212-8.

90. Menezes R, Letra A, Kim AH, Küchler EC, Day A, Tannure PN, Gomes da Motta L, Paiva KB, Granjeiro JM, Vieira AR. Studies with Wnt genes and nonsyndromic cleft lip and palate. Birth Defects Res A Clin Mol Teratol. 2010;88:995-1000.

91. Mostowska A, Hozyasz KK, Biedziak B, Wojcicki P, Lianeri M, Jagodzinski PP. Genotype and haplotype analysis of WNT genes in non-syndromic cleft lip with or without cleft palate. Eur J Oral Sci. 2012;120:1-8.

92. Lammi L, Arte S, Somer M, Jarvinen H, Lahermo P, Thesleff I, Pirinen S, Nieminen P. Mutations in AXIN2 cause familial tooth agenesis and predispose to colorectal cancer. Am J Hum Genet. 2004;74:1043-50.

93. Letra A, Menezes R, Granjeiro JM, Vieira AR. AXIN2 and CDH1 polymorphisms, tooth agenesis, and oral clefts. Birth Defects Res A Clin Mol Teratol. 2009;85:169-73

94. Menezes R, Marazita ML, Goldstein McHenry T, Cooper ME, Bardi K, Brandon C, Letra A, Martin RA, Vieira AR. AXIS inhibition protein 2, orofacial clefts and a family history of cancer. J Am Dent Assoc. 2009;140:80-4.

95. Letra A, Bjork B, Cooper ME, Szabo-Rogers H, Deleyiannis FW, Field LL, Czeizel AE, Ma L, Garlet GP, Poletta FA, Mereb JC, Lopez-Camelo JS, Castilla EE, Orioli IM, Wendell S, Blanton SH, Liu K, Hecht JT, Marazita ML, Vieira AR, Silva RM. Association of AXIN2 with non-syndromic oral clefts in multiple populations. J Dent Res. 2012;91:473-8.

96. Mostowska A, Hozyasz KK, Wójcicki P, Lasota A, Dunin-Wilczy ska I, Jagodzi ski PP. Association of DVL2 and AXIN2 gene polymorphisms with cleft lip with or without cleft palate in a polish population. Birth Defects Res A Clin Mol Teratol. 2012 Aug 6. doi: 10.1002/bdra.23056. [Epub ahead of print]

97. Ferretti E, Li B, Zewdu R, Wells V, Hebert JM, Karner C, Anderson MJ, Williams T, Dixon J, Dixon MJ, Depew MJ, Selleri L. A conserved Pbx-Wnt-p63-Irf6 regulatory module controls face morphogenesis by promoting epithelial apoptosis. Dev Cell. 2011;21:627-41

98. Song L, Li Y, Wang K, Wang YZ, Molotkov A, Gao L, Zhao T, Yamagami T, Wang Y, Gan Q, Pleasure DE, Zhou CJ. Lrp6-mediated canonical Wnt signaling is required for lip formation and fusion. Development. 2009;136:3161-71.

99. Jin YR, Han XH, Taketo MM, Yoon JK. Wnt9b-dependent FGF signaling is crucial for outgrowth of the nasal and maxillary processes during upper jaw and lip development. Development. 2012;139:1821-30.

100. Bei M, Maas R. FGFs and BMP4 induce both Msx1-independent and Msx1-dependent signaling pathways in early tooth development. Development. 1998;125:4325-33.

101. Rice R, Thesleff I, Rice DP. Regulation of Twist, Snail, and Id1 is conserved between the developing murine palate and tooth. Dev Dyn. 2005;234:28-35.

102. Matsumura K, Taketomi T, Yoshizaki K, Arai S, Sanui T, Yoshiga D, Yoshimura A, Nakamura S. Sprouty2 controls proliferation of palate mesenchymal cells via fibroblast growth factor signaling. Biochem Biophys Res Commun. 2011;404:1076-82.

103. Zhang Z, Song Y, Zhao X, Zhang X, Fermin C, Chen Y. Rescue of cleft palate in Msx1-deficient mice by transgenic Bmp4 reveals a network of BMP and Shh signaling in the regulation of mammalian palatogenesis. Development. 2002;129:4135-46.

104. Han J, Mayo J, Xu X, Li J, Bringas P Jr, Maas RL, Rubenstein JL, Chai Y. Indirect modulation of Shh signaling by Dlx5 affects the oral-nasal patterning of palate and rescues cleft palate in Msx1-null mice. Development. 2009;136:4225-33.

105. del Río A, Barrio MC, Murillo J, Maldonado E, López-Gordillo Y, Martínez-Sanz E, Martínez ML, Martínez-Álvarez C. Analysis of the presence of cell proliferation-related molecules in the Tgf-β3 null mutant mouse palate reveals misexpression of EGF and Msx-1. Cells Tissues Organs. 2011;193:135-50.

106. Ogawa T, Kapadia H, Feng JQ, Raghow R, Peters H, D'Souza RN. Functional consequences of interactions between Pax9 and Msx1 genes in normal and abnormal tooth development. J Biol Chem. 2006;281:18363-9.

107. Kong H, Wang Y, Patel M, Mues G, D'Souza RN. Regulation of bmp4 expression in odontogenic mesenchyme: from simple to complex. Cells Tissues Organs. 2011;194:156-60.

108. Satokata I, Maas R. Msx1 deficient mice exhibit cleft palate and abnormalities of craniofacial and tooth development. Nat Genet. 1994;6:348-56.

109. Fernández-Llebrez P, Grondona JM, Pérez J, López-Aranda MF, Estivill-Torrús G, Llebrez-Zayas PF, Soriano E, Ramos C, Lallemand Y, Bach A, Robert B. Msx1-deficient mice fail to form prosomere 1 derivatives, subcommissural organ, and posterior commissure and develop hydrocephalus. J Neuropathol Exp Neurol. 2004;63:574-86.

110. Ramos C, Fernández-Llebrez P, Bach A, Robert B, Soriano E. Msx1 disruption leads to diencephalon defects and hydrocephalus. Dev Dyn. 2004;230:446-60.

111. Vastardis H, Karimbux N, Guthua SW, Seidman JG, Seidman CE. A human MSX1 homeodomain missense mutation causes selective tooth agenesis. Nat Genet. 1996;13:417-21.

112. van den Boogaard MJ, Dorland M, Beemer FA, van Amstel HK. MSX1 mutation is associated with orofacial clefting and tooth agenesis in humans. Nat Genet. 2000;24:342-3.

113. Lidral AC, Romitti PA, Basart AM, Doetschman T, Leysens NJ, Daack-Hirsch S, Semina EV, Johnson LR, Machida J, Burds A, Parnell TJ, Rubenstein JL, Murray JC. Association of MSX1 and TGFB3 with nonsyndromic clefting in humans. Am J Hum Genet. 1998;63:557-68.

114. Romitti PA, Lidral AC, Munger RG, Daack-Hirsch S, Burns TL, Murray JC. Candidate genes for nonsyndromic cleft lip and palate and maternal cigarette smoking and alcohol consumption: evaluation of genotype-environment interactions from a population-based case-control study of orofacial clefts. Teratology. 1999;59:39-50.

115. Jugessur A, Lie RT, Wilcox AJ, Murray JC, Taylor JA, Saugstad OD, Vindenes HA, Abyholm F. Variants of developmental genes (TGFA, TGFB3, and MSX1) and their associations with orofacial clefts: a case-parent triad analysis. Genet Epidemiol. 2003;24:230-9.

116. Vieira AR, Orioli IM, Castilla EE, Cooper ME, Marazita ML, Murray JC. MSX1 and TGFB3 contribute to clefting in South America. J Dent Res. 2003;82:289-92.

117. Moreno LM, Arcos-Burgos M, Marazita ML, Krahn K, Maher BS, Cooper ME, Valencia-Ramirez CR, Lidral AC. Genetic analysis of candidate loci in non-syndromic cleft lip families from Antioquia-Colombia and Ohio. Am J Med Genet A. 2004 Mar 1;125A(2):135-44.

118. Schultz RE, Cooper ME, Daack-Hirsch S, Shi M, Nepomucena B, Graf KA, O'Brien EK, O'Brien SE, Marazita ML, Murray JC. Targeted scan of fifteen regions for nonsyndromic cleft lip and palate in Filipino families. Am J Med Genet A. 2004;125A:17-22.

119. Suazo J, Santos JL, Carreño H, Jara L, Blanco R. Linkage disequilibrium between MSX1 and non-syndromic cleft lip/palate in the Chilean population. J Dent Res. 2004;83:782-5.

120. Suzuki Y, Jezewski PA, Machida J, Watanabe Y, Shi M, Cooper ME, Viet le T, Nguyen TD, Hai H, Natsume N, Shimozato K, Marazita ML, Murray JC. In a Vietnamese population, MSX1 variants contribute to cleft lip and palate. Genet Med. 2004;6:117-25.

121. Tongkobpetch S, Siriwan P, Shotelersuk V. MSX1 mutations contribute to nonsyndromic cleft lip in a Thai population. J Hum Genet. 2006;51:671-6.

122. Otero L, Gutiérrez S, Cháves M, Vargas C, Bérmudez L. Association of MSX1 with nonsyndromic cleft lip and palate in a Colombian population. Cleft Palate Craniofac J. 2007;44:653-6.

123. Park J, Park BY, Kim HS, Lee JE, Suh I, Nam CM, Kang DR, Kim S, Yun JE, Go EN, Jee SH, Beaty TH. MSX1 polymorphism associated with risk of oral cleft in Korea: evidence from case-parent trio and case-control studies.Yonsei Med J. 2007;48:101-8.

124. Ingersoll RG, Hetmanski J, Park JW, Fallin MD, McIntosh I, Wu-Chou YH, Chen PK, Yeow V, Chong SS, Cheah F, Sull JW, Jee SH, Wang H, Wu T, Murray T, Huang S, Ye X, Jabs EW, Redett R, Raymond G, Scott AF, Beaty TH. Association between genes on chromosome 4p16 and non-syndromic oral clefts in four populations. Eur J Hum Genet. 2010;18:726-32.

125. Jagomägi T, Nikopensius T, Krjutskov K, Tammekivi V, Viltrop T, Saag M, Metspalu A. MTHFR and MSX1 contribute to the risk of nonsyndromic cleft lip/palate. Eur J Oral Sci. 2010;118:213-20.

126. Nikopensius T, Jagomägi T, Krjutskov K, Tammekivi V, Saag M, Prane I, Piekuse L, Akota I, Barkane B, Krumina A, Ambrozaityte L, Matuleviciene A, Kucinskiene ZA, Lace B, Kucinskas V, Metspalu A. Genetic variants in COL2A1, COL11A2, and IRF6 contribute risk to nonsyndromic cleft palate. Birth Defects Res A Clin Mol Teratol. 2010;88:748-56.

127. Vieira AR, Castillo Taucher S, Aravena T, Astete C, Sanz P, Tastets ME, Monasterio L, Murray JC. [Mutational analysis of the muscle segment homeobox gene 1 (MSX1) in Chilean patients with cleft lip/palate]. Rev Med Chil. 2004;132:816-22.

128. Jezewski PA, Vieira AR, Nishimura C, Ludwig B, Johnson M, O'Brien SE, Daack-Hirsch S, Schultz RE, Weber A, Nepomucena B, Romitti PA, Christensen K, Orioli IM, Castilla EE, Machida J, Natsume N, Murray JC. Complete sequencing shows a role for MSX1 in non-syndromic cleft lip and palate. J Med Genet. 2003;40:399-407.

129. Salahshourifar I, Halim AS, Wan Sulaiman WA, Zilfalil BA. Contribution of MSX1 variants to the risk of non-syndromic cleft lip and palate in a Malay population. J Hum Genet. 2011;56:755-8.

130. Lidral AC, Reising BC. The role of MSX1 in human tooth agenesis. J Dent Res. 2002;81:274-8.

131. Vieira AR, Avila JR, Daack-Hirsch S, Dragan E, Félix TM, Rahimov F, Harrington J, Schultz RR, Watanabe Y, Johnson M, Fang J, O'Brien SE, Orioli IM, Castilla EE, Fitzpatrick DR, Jiang R, Marazita ML, Murray JC. Medical sequencing of candidate genes for nonsyndromic cleft lip and palate.PLoS Genet. 2005 Dec;1(6):e64.

132. Huang YQ, Ma J, Ma M, Deng Y, Li YD, Ren HW, Zhao GZ, Guo SS, Wang YY, Zhang GX, Shi B. Association between MSX1 variants and oral clefts in Han Chinese in western China. DNA Cell Biol. 2011;30:1057-61.

133. Bergendal B, Klar J, Stecksén-Blicks C, Norderyd J, Dahl N. Isolated oligodontia associated with mutations in EDARADD, AXIN2, MSX1, and PAX9 genes. Am J Med Genet A. 2011 Jul;155A(7):1616-22.

134. De Muynck S, Schollen E, Matthijs G, Verdonck A, Devriendt K, Carels C. A novel MSX1 mutation in hypodontia. Am J Med Genet A. 2004;128A:401-3.

135. van den Boogaard MH, Rob H. J. M. Gooskens RHJM, Marijn Créton M, Rutger-Jan Nievelstein R-J, Hans Kristian Ploos van Amstel JK, Dick Lindhout L . Identification of a missense mutation in the MSX1 homeodomain, in a patient with hydrocephaly. Eur J Hum Gen. submitted

136. Liang J, Zhu L, Meng L, Chen D, Bian Z. Novel nonsense mutation in MSX1 causes tooth agenesis with cleft lip in a Chinese family. Eur J Oral Sci. 2012;120:278-82.

137. Mostowska A, Biedziak B, Trzeciak WH. A novel c.581C>T transition localized in a highly conserved homeobox sequence of MSX1: is it responsible for oligodontia? J Appl Genet. 2006;47(2):159-64.

138. van den Boogaard M-JH, Babajko S, Fernandez I, Veenstra-Knol HE, Daniel van der Meulen B, Lindhout D, Oosterwijk JC, Berdal A, Ploos van Amstel JK. MSX1 antisense as a potential modulator of the phenotype in two cheilo-gnatho-palatoschisis families with a MSX1 mutation. Manuscript in preparation.

139. Jumlongras D, Bei M, Stimson JM, Wang WF, DePalma SR, Seidman CE, Felbor U, Maas R, Seidman JG, Olsen BR. A nonsense mutation in MSX1 causes Witkop syndrome. Am J Hum Genet. 2001;69:67-74.

140. van den Boogaard MJ, Créton M, Bronkhorst Y, van der Hout A, Hennekam E, Lindhout D, Cune M, Ploos van Amstel HK. Mutations in WNT10A are present in more than half of isolated hypodontia cases. J Med Genet. 2012;49:327-31.

141. Chishti MS, Muhammad D, Haider M, Ahmad W. A novel missense mutation in MSX1 underlies autosomal recessive oligodontia with associated dental anomalies in Pakistani families. J Hum Genet. 2006;51:872-8.

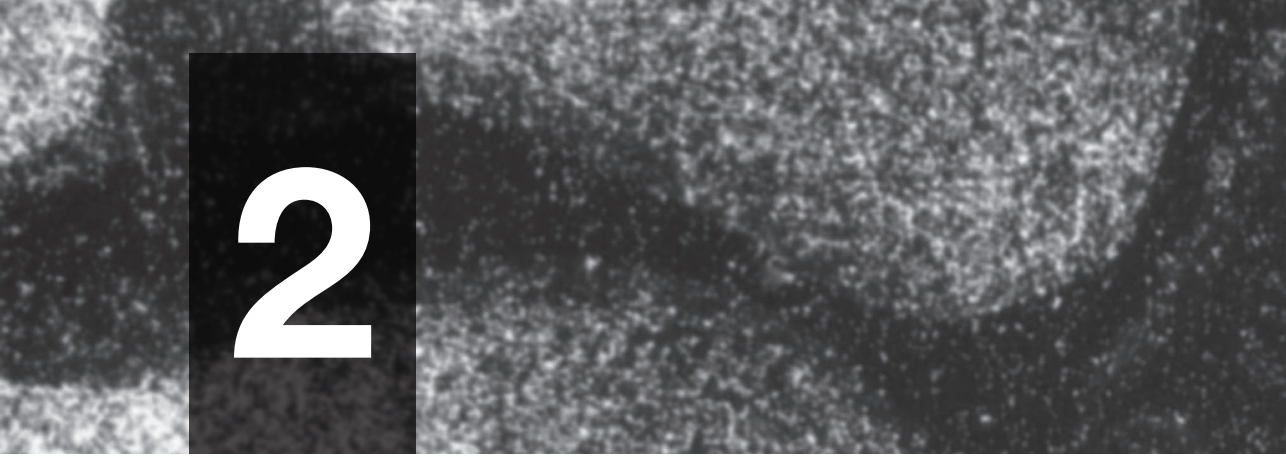
142. Xuan K, Jin F, Liu YL, Yuan LT, Wen LY, Yang FS, Wang XJ, Wang GH, Jin Y. Identification of a novel missense mutation of MSX1 gene in Chinese family with autosomal-dominant oligodontia. Arch Oral Biol. 2008;53:773-9

143. Mostowska A, Biedziak B, Jagodzinski PP. Novel MSX1 mutation in a family with autosomal-dominant hypodontia of second premolars and third molars. Arch Oral Biol. 2012;57:790-5.

144. Li WL, Cui JJ, Fang QY, Mei LX. [A novel mutation of MSX1 gene in a Chinese pedigree with oligodontia]. Zhonghua Kou Qiang Yi Xue Za Zhi. 2008;43:157-9.

145. Kim JW, Simmer JP, Lin BP, Hu JC. Novel MSX1 frameshift causes autosomal-dominant oligodontia. J Dent Res. 2006;85:267-71.

146. Nieminen P, Kotilainen J, Aalto Y, Knuutila S, Pirinen S, Thesleff I. MSX1 gene is deleted in Wolf-Hirschhorn syndrome patients with oligodontia. J Dent Res. 2003;82:1013-7.



2

Chapter 2

Orofacial Clefting and associated anomalies

A systematic review of associated structural and chromosomal defects in oral clefts: when is prenatal genetic analysis indicated?

Maarse W, Rozendaal AM, Pajkrt E, Vermeij-Keers C, Mink van der Molen AB, van den Boogaard MJ.

Published in: J Med Genet. 2012 Aug;49(8):490-8.

Abstract

Background

Oral clefts—comprising cleft lip (CL), cleft lip with cleft palate (CLP), and cleft palate (CP)—are being diagnosed prenatally more frequently. Consequently, the need for accurate information on the risk of associated anomalies and chromosomal defects to aid in prenatal counselling is rising. This systematic review was conducted to investigate the prenatal and postnatal prevalence of associated anomalies and chromosomal defects related to cleft category, thereby providing a basis for prenatal counselling and prenatal invasive diagnostics.

Methods

Online databases were searched for prenatal and postnatal studies on associated anomalies and chromosomal defects in clefts. Data from the literature were complemented with national validated data from the Dutch Oral Cleft Registry.

Results

Twenty studies were included: three providing prenatal data, 13 providing postnatal data, and four providing both. Data from prenatal and postnatal studies showed that the prevalence of associated anomalies was lowest in CL (0–20.0% and 7.6–41.4%, respectively). For CLP, higher frequencies were found both prenatally (39.1–66.0%) and postnatally (21.1–61.2%). Although CP was barely detectable by ultrasound, it was the category most frequently associated with accompanying defects in postnatal studies (22.2–78.3%). Chromosomal abnormalities were most frequently seen in association with additional anomalies. In the absence of associated anomalies, chromosomal defects were found prenatally in CLP (3.9%) and postnatally in CL (1.8%, 22q11.2 deletions only), CLP (1.0%) and CP (1.6%).

Conclusions

Prenatal counselling regarding prognosis and risk of chromosomal defects should be tailored to cleft category, and more importantly to the presence/absence of associated anomalies. Irrespective of cleft category, clinicians should advise invasive genetic testing if associated anomalies are seen prenatally. In the absence of associated anomalies, prenatal conventional karyotyping is not recommended in CL, although array comparative genomic hybridisation should be considered. In presumed isolated CLP or CP, prenatal invasive testing, preferably by array based methods, is recommended.

Introduction

Oral clefts—one of the most common congenital malformations in humans—arise in approximately 1 in 700 live births. [1] It is has been well established that, although clefts can be isolated anomalies, they are frequently associated with other congenital anomalies, often as part of a syndrome or chromosomal defect. Oral clefts are traditionally subdivided into two categories: cleft lip with or without cleft palate (CL±P), and cleft palate only (CP). [1] However, recent studies have emphasised subdivision into three categories: cleft lip only (CL), cleft lip with cleft palate (CLP), and CP, because of differences concerning embryologic development, prevalence, risk factors, and associations with other congenital anomalies. [2–5] Although many studies have also included median cleft lip or atypical facial clefts as oral clefts, [6–8] these anomalies should be considered as separate craniofacial anomalies because of their different pathogenesis. [9, 10]

As a result of advances in transabdominal two dimensional ultrasound technology and its routine use in obstetric practice, oral clefts with or without associated anomalies are being diagnosed prenatally more frequently. [11] Detection rates—predominantly on CL±P—increased from approximately 5% in the early 1980s to over 26% in the late 1990s, [12] and they are as high as 65% today. [13] Consequently, there is an increasing need for accurate information to aid in prenatal counselling. When informing parents on outcome and prognosis, the category of cleft as well as the presence of other congenital anomalies is crucial. In particular, the identification of an underlying chromosomal defect will influence prenatal counselling and the management of the pregnancy significantly. However, in clinical practice there is often discussion on whether further invasive tests should be performed prenatally to identify chromosomal defects. [14] It is unknown whether invasive diagnostics should be offered in all identified cleft cases, or should be limited to specific cleft categories or the presence of associated anomalies.

To allow informed decisions on invasive prenatal diagnostics, clinicians and parents need to be informed about the prevalence of associated anomalies and underlying chromosomal defects in clefts. However, the reported rates in prenatal cleft populations vary greatly between studies. [6–8, 13–16] Furthermore, these findings may reflect selection bias,[17] as cases that are more likely to be diagnosed prenatally tend to be the more severe cases with associated anomalies and chromosomal defects. [11] Nowadays, increasing numbers of isolated clefts—not accompanied by growth retardation or other

prenatal complications—are identified in utero. [11] Therefore, both prenatal and postnatal studies have to be interpreted in order to provide accurate information on the frequencies of associated anomalies and the underlying chromosomal defects for future prenatal cleft populations.

This systematic review presents a comprehensive summary of the literature and complementary Dutch registry data on prenatal and postnatal findings of associated anomalies and chromosomal defects related to cleft category. The aim of this study was to provide a basis for prenatal counselling of future parents and to advise on invasive genetic diagnostics in prenatally detected oral clefts.

Methods

Literature search

In August 2011, the PubMed database was systematically searched using the search string '(cleft) AND (abnormalities OR anomalies) AND (chromosomal OR syndrome)'. The search was limited to articles published in English after 31 December 1994. This restriction was applied because technologies to identify specific syndrome diagnoses and chromosomal abnormalities have been developed relatively recent. For example, fluorescence in situ hybridisation (FISH) was introduced in clinical practice in the early 1990s, enabling the detection of specific microdeletions. [18, 19] Consequently, studies published before 1995 may have reported relatively underestimated rates of associated anomalies and chromosomal defects.

The titles and abstracts of the citations were screened independently by two reviewers (MJB and WM) to identify potentially relevant papers for which full text publications were retrieved. Additional studies were found by crosschecking references. Studies were included if they presented data on oral clefts that were analysed prenatally and/or postnatally for associated anomalies and chromosomal defects, the latter preferably verified by karyotype. To ensure the quality and prevent our prenatal analysis from significant underreporting, we excluded prenatal studies in which several obvious structural defects (eg, anencephaly or holoprosencephaly) had been missed by ultrasound. Because of the ethnic variation in prevalence of clefts and their associated anomalies, [1, 17] studies evaluating non-Caucasian populations (eg, Asian populations) were excluded to keep a homogeneous study population.

Complementary data

Comparison of the existing literature on congenital anomalies and chromosomal defects associated with oral clefts is restricted, particularly due to differences in methodology. For example, there is a considerable variation in definitions and classifications of clefts and their accompanying defects, as well as in sample sizes, data sources, methods of data collection, and follow-up periods between studies. [17] For this reason, we complemented our review of postnatal studies with national data from the Dutch Oral Cleft Registry (NVSCA). Since 1997, the 15 Dutch cleft palate teams have registered oral clefts and their associated anomalies, using a unique detailed recording system based on the embryology of the head and neck area. Because major as well as minor anomalies (including dysmorphic features) are recorded in detail, the NVSCA data can be fitted into any existing classification and are highly applicable for comparison with other studies. Moreover, a selection of registry data has recently been validated and completed by a review of medical data, after a median follow-up period of 5 years. [20–22] This selection of validated data was used to complement our analysis on associated anomalies and chromosomal defects in postnatally detected clefts. [22] In addition, the annual NVSCA reports 1997–2010 were used to provide an inventory of the different syndromes and chromosomal defects that had been identified postnatally. [23, 24] The methods of registration and validation have been described in detail elsewhere. [5, 20–22]

Data analysis

Data on associated anomalies and chromosomal defects were extracted from the selected articles and subdivided according to the three cleft categories: CL, CLP, and CP. Also, the validated and completed NVSCA data were further analysed according to these three categories. [22] For studies not distinguishing CL and CLP, the category of CL±P was used. Median cleft lip and atypical facial clefts were excluded because of their different pathogenesis. [9, 10]

For all cleft categories, frequencies of associated congenital anomalies and chromosomal defects were deducted from the reported data and presented in numbers and percentages. For studies providing karyotype information for isolated and/or associated cases, we calculated separate rates of chromosomal defects among isolated (if available) and associated clefts. [6–8, 13–15, 22, 25–27] If studies did not provide numbers of karyotyped cases, but reported routine karyotyping of associated clefts (as in daily practice), we assumed that the majority of associated clefts were karyotyped. [13, 22] Likewise, if chromosomal defects were reported from retrospective registry data without information about the presence or

absence of associated anomalies, we assumed that chromosomal analysis had been performed in associated cases only. [16, 26, 28–31] If no specific data on chromosomal anomalies were available for the three cleft categories, [32–36] total rates of associated anomalies were calculated. Theoretically, these numbers might also include chromosomal anomalies detected in isolated clefts, without other congenital anomalies. Where possible, prevalence data were also subdivided according to unilateral and bilateral clefts. [6–8, 13, 14, 25]

To specify the detected chromosomal anomalies, we made an inventory of the different syndromes and chromosomal defects that had been identified in clefts prenatally and/or postnatally. Due to great differences in methodology, [17] we were not able to perform a meta-analysis with these data. To provide more information about the reviewed studies and to illustrate the differences, we summarised the various study characteristics and designs, including the inclusion criteria and definitions of clefts and accompanying defects, as well as the sample sizes, data sources, and methods of data collection.

Results

The literature search yielded 9540 citations. Initial screening by title identified 88 potentially relevant abstracts, including 20 studies meeting the inclusion criteria. Subsequently, one of these studies was excluded because obvious structural anomalies (eg, lobar holoprosencephaly and severe congenital heart anomalies) had been missed prenatally, which raised doubts about the quality of the performed prenatal ultrasounds. [37]

Including the NVSCA study, [22] the remaining studies comprised three studies providing relevant prenatal data, [6–8] 13 studies providing relevant postnatal data,[22, 25–36] and four studies providing both. [13–16] All studies with postnatal data had a follow-up period of at least 1 year. Although the studies of Stoll et al., [26] Vallino-Napoli et al., [29] and Walker et al. [25] presented both prenatal and postnatal data, they were included only in the postnatal analysis for divergent reasons. First, the retrospective data of Stoll et al. [26] did not allow extraction of frequencies of associated anomalies and chromosomal defects among prenatally detected clefts. Second, Vallino-Napoli et al. [29] reported data on pregnancy outcome, but the prenatally detected cleft cases could not be identified from their data. Finally, Walker et al. [25] evaluated anomalies that could theoretically have been detected by ultrasound instead of those that had actually been detected. The latter were not separately discussed in their paper. The various study characteristics and designs of the reviewed studies are presented in Supplementary Table 2.1.

Table 2.1 Summary of published prevalence data on associated anomalies and chromosomal defects in prenatally and postnatally detected oral clefts

Table 2.1

Study type	Associated anomalies % (n)	Chromosomal defects		
		Isolated clefts* % (n)	Associated clefts* % (n)	Total clefts* % (n)
Prenatal studies				
CL				
Nyberg *et al* [6]†	20.0 (1/5)		0.0 (0/1)‡	0.0 (0/5)
Berge *et al* [7]†	0.0 (0/3)	0.0 (0/3)	0.0 (0/0)	0.0 (0/3)
Maarse *et al* [13]†	13.3 (2/15)		50.0 (1/2)	6.7 (1/15)
Total	13.0 (3/23)	0.0 (0/3)	33.3 (1/3)	4.3 (1/23)
CLP				
Nyberg *et al* [6]†	45.7 (16/35)	5.3 (1/19)	50.0 (8/16)	25.7 (9/35)
Berge *et al* [7]†	66.0 (35/53)	0.0 (0/18)	68.6 (24/35)	46.3 (25/54)§
Maarse *et al* [13]†	39.1 (9/23)	7.1 (1/14)	66.7 (6/9)	30.4 (7/23)
Total	54.0 (60/111)	3.9 (2/51)	63.3 (38/60)	36.7 (41/112)§
CL±P				
Perrotin *et al* [8]†	35.7 (20/56)	0.0 (0/36)	55.0 (11/20)‡	19.6 (11/56)
Offerdal *et al* [15]	57.1 (20/35)		40.0 (8/20)	22.9 (8/35)
Russell *et al* [16]	51.7 (15/29)		33.3 (5/15)‡¶	17.2 (5/29)
Gillham *et al* [14]†	17.2 (26/151)	0.0 (0/122)	34.6 (9/26)	6.0 (9/151)
Total	29.9 (81/271)	0.0 (0/158)	40.7 (33/81)	12.2 (33/271)
CP				
Berge *et al* [7]†	100.0 (2/2)		100.0 (2/2)‡	100.0 (2/2)‡
Postnatal studies				
CL				
Kallen *et al* [28]	10.4 (212 /2029)		10.4 (22/212)¶	1.1 (22/2029)
Milerad *et al* [33]	8.0 (13/163)**			
Walker *et al* [25]††	8.3 (7/84)		14.3 (1/7)	1.2 (1/84)
Calzolari *et al* [30]	13.6 (245/1806)		13.1 (32/245)¶	1.8 (32/1806)
Tan *et al* [31]	11.9 (8/67)		12.5 (1/8)¶	1.5 (1/67)
Maarse *et al* [13]	11.8 (2/17)		0.0 (0/2)	0.0 (0/17)
Rittler *et al* [27]	7.6 (9/119)	1.8 (2/110)‡‡	22.2 (2/9)	3.4 (4/119)
Rozendaal *et al* [22]	41.4 (29/70)		0.0 (0/29)	0.0 (0/70)
Total	12.1 (525/4355)	1.8 (2/110)	11.3 (58/512)	1.4 (60/4192)
CLP				
Kallen *et al* [28]	25.3 (819/3232)		24.5 (201/819)¶	6.2 (201/3232)
Milerad *et al* [33]	28.0 (60/214)**			
Walker *et al* [25]††	24.6 (44/179)		31.8 (14/44)	7.8 (14/179)
Calzolari *et al* [30]	23.8 (693/2913)		22.1 (153/693)¶	5.3 (153/2913)

Table 2.1 continued

	Associated anomalies % (n)	Chromosomal defects		
Study type		Isolated clefts* % (n)	Associated clefts* % (n)	Total clefts* % (n)
CLP				
Tan *et al* [31]	23.2 (22/95)		13.6 (3/22)¶	3.2 (3/95)
Maarse *et al* [13]	21.1 (4/19)		25.0 (1/4)	5.3 (1/19)
Rittler *et al* [27]	23.5 (93/395)	1.0 (3/302)‡‡	28.0 (26/93)	7.3 (29/395)
Rozendaal *et al* [22]	61.2 (60/98)		5.0 (3/60)	3.1 (3/98)
Total	25.1 (1795/7145)	1.0 (3/302)	23.1 (401/1735)	5.8 (404/6931)
CL±P				
Drushel, 1996 [32]	29.2 (467/1599)**			
DeRoo *et al* [34]	22.9 (64/280)**			
Shaw *et al* 35	60.2 (2453/4072)**			10.3 (419/4072)
Vallino-Napoli *et al* [29]	25.1 (299/1189)		33.8 (101/299)¶	8.5 (101/1189)
Stoll *et al* [26]	27.9 (109/390)		33.0 (36/109)¶	9.2 (36/390)
Russell *et al* [16]	37.0 (47/127)		34.0 (16/47)¶	12.6 (16/127)
Offerdal *et al* [15]	33.3 (22/66)		4.5 (1/22)	1.5 (1/66)
Beriaghi *et al* [36]	26.4 (157/595)**			
Gillham *et al* [14]	7.2 (16/222)	0.0 (0/206)	6.3 (1/16)	0.5 (1/222)
Total	42.6 (3634/8540)	0.0 (0/206)	31.4 (155/493)	9.5 (574/6066)
CP				
Drushel 1996 [32]	43.6 (517/1187)**			
Kallen *et al* [28]	29.0 (732/2527)		18.3 (134/732)¶	5.3 (134/2527)
Milerad *et al* [33]	22.2 (53/239)**			
DeRoo *et al* [34]	64.9 (144/222)**			
Shaw *et al* [35]	71.1 (1665/2343)**			10.6 (249/2343)
Vallino-Napoli *et al* [29]	41.7 (347/833)		21.0 (73/347)¶	8.8 (73/833)
Stoll *et al* [26]	47.9 (125/261)		14.4 (18/125)¶	6.9 (18/261)
Russell *et al* [16]	53.1 (52/98)		11.5 (6/52)¶	6.1 (6/98)
Offerdal *et al* [15]	50.0 (10/20)		30.0 (3/10)	15.0 (3/20)
Tan *et al* [31]§§	23.1 (27/117)		29.6 (8/27)¶	6.8 (8/117)
Beriaghi *et al* [36]	38.7 (206/532)**			
Gillham *et al* [14]	26.6 (67/252)			
Maarse *et al* [13]	52.9 (9/17)		0.0 (0/9)	0.0 (0/17)
Rittler *et al* [27]	42.3 (83/196)	0.0 (0/113)	12.0 (10/83)	5.1 (10/196)
Rozendaal *et al* [22]	78.3 (54/69)	13.3 (2/15)	16.7 (9/54)	15.9 (11/69)
Total	45.9 (4091/8913)	1.6 (2/128)	18.1 (261/1439)	7.9 (512/6481)

Table 2.1 Summary of published prevalence data on associated anomalies and chromosomal defects in prenatally and postnatally detected oral clefts

CL, cleft lip only; CLP, cleft lip with cleft palate; CL±P, cleft lip with or without cleft palate; CP, cleft palate only.
Blank entry: data were not available or could not be deducted.
*Information on karyotype not available for all clefts, unless stated differently (see also supplementary table 1). Therefore, inclusion of undetected chromosomal defects cannot be ruled out. Null values were given only if information about chromosomal analysis was reported.

†Median cleft lip and atypical facial clefts were excluded because of their different pathogenesis.
‡Karyotype available for all clefts.
§For one case with a chromosomal defect, data on associated anomalies were not available.
¶Retrospective analysis of data from birth or birth defect registries. Although not specifically mentioned whether chromosomal defects were accompanied by additional anomalies, we assumed that karyotype analysis had been performed only in associated clefts (as is generally done in clinical practice).
**No specific data given about type of associated anomalies, including chromosomal defects. Therefore, inclusion of chromosomal defects in isolated clefts cannot be ruled out.
††Because of limited data, chromosomal defects among isolated clefts not given.
‡‡Including deletions 22q11.2 identified by array comparative genomic hybridisation.
§§Pierre Robin sequence excluded.

Prenatally detected associated anomalies and chromosomal defects

In the seven prenatal studies, a total of 407 fetuses with oral clefts were analysed. [6–8, 13–16] The prevalence of associated anomalies and chromosomal defects in prenatally detected clefts is summarised according to cleft category in Table 2.1. In the CL category, three out of 23 fetuses had associated anomalies, comprising a cardiac defect with a situs inversus,[6] an umbilical hernia, and a clubfoot.[13] One of these three CL cases had a chromosomal defect (trisomy 18). [13] CLP showed the highest prevalence of associated anomalies (54.0%, range 39.1–66.0%). For studies that grouped CL and CLP together as CL±P, the prevalence was somewhat lower (29.9%, range 17.2–57.1%). Only one study evaluated prenatally detected CP cases (n=2); both cases had associated anomalies as well as an underlying chromosomal defect. [7] In addition to the three cleft categories, studies distinguishing unilateral and bilateral clefts generally found a higher prevalence of associated anomalies and chromosomal defects in bilateral than in unilateral CLP or CL±P (Table 2.2).

Analysis of chromosomal defects in isolated and associated clefts revealed that almost all chromosomal defects were associated with other congenital anomalies or ultrasound markers, such as intrauterine growth retardation (97.4%, 74/76; one case with a chromosomal defect not included, as information on associated anomalies was not available, table 2.1). [6] For only two cases with chromosomal defects, no accompanying defects were found by ultrasound; one case showed a mosaic trisomy 22 [6] and the other had a trisomy 18. [13] Consequently, the prevalence of chromosomal defects in cases with associated clefts was 50.7% (74/146), while it was 0.9% (2/212) in cases with formerly presumed isolated clefts. In studies specifying the detected chromosomal abnormalities, trisomy 13 (56.3%, 36/64) and trisomy 18 (29.7%, 19/64) were the most commonly observed defects. [6–8, 13, 14] Offerdal et al. [15] and Russell et al[16] did not specify prenatally identified chromosomal defects in their study (n=8 and n=5, respectively).

Postnatally detected associated anomalies and chromosomal defects

Seventeen studies analysed a total of 28 953 infants with oral clefts. [13–16, 22, 25–36] Table 2.1 shows the prevalence of associated anomalies and chromosomal defects in postnatally detected clefts. Similar to the prenatal analysis, postnatal studies showed that CL was less frequently associated with accompanying defects than the other two cleft categories. The prevalence of associated anomalies in CL was approximately 10%, except for the study of Rozendaal et al (41.4%). [22] For CLP and CL±P, most studies showed a prevalence of approximately 25%. However, the studies of Shaw et al. [35] and Rozendaal et al. [22] found a prevalence of about 60%. All studies reported that CP was

the category most frequently associated with additional anomalies (45.9%; range 22.2–78.3%). When analysing the underlying chromosomal defects, the prevalence was highest in CL±P (9.5%, range 0.5–12.6%). The lowest prevalence of chromosomal defects was found in CL (1.4%, range 0–3.4%). Studies distinguishing unilateral and bilateral clefts showed a higher prevalence among bilateral than unilateral CLP (Table 2.2).

Analysis of chromosomal defects in isolated and associated clefts revealed that almost all chromosomal abnormalities were found in association with additional anomalies. Only two studies found chromosomal defects in isolated clefts. In the study of Rittler et al., [27] information was available for 58% (108/185) of the isolated cleft cases (supplementary table 2.1). They found diagnostic evidence for chromosomal defects in 1.8% (2/110) of the CL cases (both having a deletion 22q11.2), and in 1.0% (3/302) of the CLP cases. The latter three cases showed a deletion 22q11.2, a 46,X,del(X)(q1.3), and a 46,XY,add(15)

Table 2.2 Summary of published prevalence data on associated anomalies and chromosomal defects in prenatally and postnatally detected unilateral and bilateral oral clefts

	Associated anomalies		
			Isolated clefts*
Study type	**Unilateral % (n)**	**Bilateral % (n)**	**Unilateral % (n)**
Prenatal studies			
CLP			
Nyberg et al [6]†	40.0 (6/15)	55.0 (10/20)	11.1 (1/9)
Berge et al [7]†	52.0 (13/25)	78.6 (22/28)	0.0 (0/12)
Maarse et al [13]†	35.3 (6/17)	50.0 (3/6)	0.0 (0/11)
Total	45.6 (26/57)	64.8 (35/54)	4.8 (1/32)
CL±P			
Perrotin et al [8]†	24.1 (7/29)	48.1 (13/27)	0.0 (0/22)
Gillham et al [14]†	15.5 (18/116)	22.9 (8/35)	0.0 (0/98)
Total	17.2 (25/145)	33.9 (21/62)	0.0 (0/110)
Postnatal studies			
CL			
Walker et al [25]¶	8.3 (6/72)	8.3 (1/12)	
CLP			
Walker et al [25]¶	20.5 (23/112)	31.3 (21/67)	

CL, cleft lip only; CLP, cleft lip with cleft palate; CL±P, cleft lip with or without cleft palate; CP, cleft palate only.
Blank entry: data were not available or could not be deducted.
*Information on karyotype not available for all clefts, unless stated differently (see also supplementary table 1). Therefore, inclusion of undetected chromosomal defects cannot be ruled out.
Null values were given only if information about chromosomal analysis was reported.

(p11). As the 22q11.2 deletions were identified with array comparative genomic hybridisation (array CGH) during follow-up, the rate of chromosomal defects detected by standard karyotyping was 0% (0/110) and 0.7% (2/302) for CL and CLP, respectively. Although the rate of karyotyped cases was not known in the study of Rozendaal et al., [22] they found that two out of 15 isolated CP cases had chromosomal defects (trisomy 21 and 46,XY,add(14)(p), respectively). In both cases, the identification of the chromosomal abnormality was delayed due to the absence of additional congenital anomalies. An inventory of the reported chromosomal defects, non-chromosomal syndromes, and other diagnoses associated with prenatally and/or postnatally detected clefts is provided in Supplementary Table 2.2.

Table 2.2

	Chromosomal defects			
	Associated clefts*		Total clefts*	
Bilateral % (n)	Unilateral % (n)	Bilateral % (n)	Unilateral % (n)	Bilateral % (n)
0.0 (0/10)	33.3 (2/6)	54.4 (6/10)	20.0 (3/15)	30.0 (6/20)
0.0 (0/6)	61.5 (8/13)	72.7 (16/22)	32.0 (8/25)	58.6 (17/29)§
33.3 (1/3)	66.7 (4/6)	66.7 (2/3)	23.5 (4/17)	50.0 (3/6)
5.3 (1/19)	57.7 (15/25)	68.6 (24/35)	26.3 (15/57)	47.3 (26/55)§
0.0 (0/14)	57.1 (4/7)‡	53.8 (7/13)‡	13.8 (4/29)	25.9 (7/27)
0.0 (0/27)	33.3 (6/18)	37.5 (3/8)	5.2 (6/116)	8.6 (3/35)
0.0 (0/41)	40.0 (10/25)	47.6 (10/21)	6.9 (10/145)	16.1 (10/62)
	16.7 (1/6)	0.0 (0/1)	1.4 (1/72)	0.0 (0/12)
	21.7 (5/23)	42.9 (9/21)	4.5 (5/112)	13.4 (9/67)

†Median cleft lip and atypical facial clefts were excluded because of their different pathogenesis.
‡Karyotype available for all clefts.
§For one case with a chromosomal defect, data on associated anomalies were not available.
¶Because of limited data, chromosomal defects among isolated clefts not given

Discussion

This systematic review assessed the association of prenatally and postnatally detected oral clefts with other congenital anomalies and underlying chromosomal defects, thereby providing a basis for prenatal counselling and well-informed decisions on invasive prenatal diagnostics in clefts. We demonstrated that the prevalence of associated structural and chromosomal defects is evidently related to cleft category. Although varying in study characteristics and designs, both prenatal and postnatal studies showed a higher frequency of associated anomalies and chromosomal defects in CLP and CP than in CL. For all cleft categories, chromosomal defects were almost always seen in association with additional congenital anomalies. Therefore, the presence of additional anomalies on ultrasound is the most important predictor of underlying chromosomal defects in fetuses with oral clefts.

Methodological issues

The use of both prenatal and postnatal studies—including detailed Dutch registry data—gave our study its main strength. It allowed us to provide a more reliable and representative basis for prenatal counselling and genetic testing than when only prenatal studies were evaluated. As the proportion of detected isolated clefts in prenatal populations is rising, previous prenatal studies may not have provided representative samples of current/future prenatal cleft populations. Overall, prenatal rates of associated anomalies and chromosomal defects may have been too high, because associated clefts are more likely to be detected by ultrasound than isolated clefts, [11] and some prenatal cases never reach term due to lethal anomalies or termination of pregnancy (TOP). [16, 25, 26, 29] Another advantage of our evaluation of postnatal studies is that congenital anomalies not detected by ultrasound were also included. In particular, studies with a longer follow-up allowed us to consider minor anomalies and features that become more evident later in life. [22] For example, individuals with the velo-cardio-facial (VCF) syndrome (22q11.2 deletion) are often diagnosed at school age when speech and learning difficulties become evident, unless a cardiac defect manifests earlier. [38] Our study was also strengthened by its focus on clinical genetic aspects. If provided, karyotype information was evaluated and separate rates of chromosomal defects among isolated (if available) and associated clefts were calculated. Besides these prevalence rates, we also composed an inventory of the different syndromes and chromosomal defects in prenatally and/or postnatally detected clefts reported by the reviewed studies and complemented with Dutch registry data, thereby specifying the detected anomalies (supplementary table 2.2).

However, combining results from different studies also had its limitations, mainly due to methodological issues. As summarised in supplementary table 2.1, we found many differences in study characteristics and designs between the reviewed studies, which are in line with those reported by Wyszynski et al. [17] The most important issue was non-uniform subdivision of oral clefts. Some studies distinguished CL and CLP [6, 7, 13, 25, 27, 28, 30, 31, 33] while others grouped them as CL±P. [8, 14–16, 26, 29, 32, 34–36] Together with previous studies, [2–5] our results stress the need of accurate prenatal subdivision into three categories (CL, CLP, and CP). Obviously, analysing CL and CLP as one group will result in different frequencies of associated anomalies and chromosomal defects than when they are analysed separately. Unfortunately, prenatal distinction between CL and CLP can be limited because prenatal identification of involvement of the palate is still challenging. [11, 14] For this reason, data on prenatally detected CP were limited in the current study. However, there is evidence of improvements in imaging, as well as in experience in detection and interpretation of subtle signs on ultrasound, [39, 40] which will progressively reduce the lower limits for detection.

Another important factor was that associated anomalies were differently defined and classified in the evaluated studies, which partly explains the wide variation in the reported rates of associated anomalies. [17] The definitions in the reviewed studies ranged from only major (structural) non-facial congenital anomalies to all anomalies, including minor congenital anomalies and ultrasound markers, such as intrauterine growth retardation (supplementary table 1). This might explain, at least partially, the relatively high rates of associated anomalies reported by Rozendaal et al., [22] who also included minor and dysmorphic features in their analysis. Although these minimal defects are hardly detected prenatally, they may be recognisable components of specific syndromes or chromosomal defects in postnatally detected clefts. [17] Similarly, the high prevalence reported by Shaw et al[35] could also partly be due to the inclusion of minor defects, as they used diagnostic codes with low specificity, including the malformation groups 'ear, face, neck' and 'upper alimentary tract'.
Another source of variation is the inconsistent definition of the Pierre Robin sequence applied in clinical practice and consequently its over- or under-reporting. [41] Some of the reviewed studies classified this condition—CP combined with micrognathia, glossoptosis and airway compromise—as isolated CP [26, 27, 33] while other studies considered it as a separate category [28, 31] or as associated CP. [11, 14, 16, 22, 29]

The reviewed studies also varied considerably in their reporting of karyotypic information (supplementary table 2.1). While some studies provided explicit information about the

number of karyotyped cases and their detected associated and chromosomal defects, [6–8, 13–15, 22, 25, 27] others reported only abnormal karyotypes, but not their associated anomalies, [16, 26, 28–31] or they did not give any specific data at al. [32–36] As a consequence, separate and complete rates of chromosomal defects could not always be obtained. Furthermore, in studies providing explicit information, chromosomal analysis was mostly performed in associated clefts only, which explains why almost all reported chromosomal defects were accompanied by additional anomalies. It is important to realise that most of these studies obtained chromosome results for just a part—and not all—of the clefts, and that the inclusion of cases with undetected chromosomal defects in their rates therefore cannot be ruled out. Besides karyotype analysis, most studies also did not report whether FISH analysis had been performed and whether microdeletions were included in the presented data. Only the studies of Tan et al., [31] Rittler et al., [27] and Rozendaal et al. [22] reported the inclusion of microdeletions or duplications, while Stoll et al. [26] included the results of FISH22q11 screening as from 1994. In contrast, Kallen et al. [28] reported not to have included microdeletions, which might have led to an underestimation of the frequency of underlying chromosomal defects. On the other hand, some studies may have overrepresented chromosomal defects in association with oral clefts due to the inclusion of sex chromosome abnormalities. For example, Stoll et al. [26] showed that 12 out of 54 abnormal karyotypes concerned abnormalities of sex chromosomes, which may be coincidental findings and not related to clefts. From the literature, no convincing evidence is provided that the most frequently detected sex chromosomal anomalies (eg, 47, XXX; and 47, XXY) are actually related to clefts.

Differences in study settings and data sources between studies (supplementary table 2.1) may also have accounted for the variation in the prevalence of associated anomalies and are possible sources of selection bias. For example, some studies were performed with data from prenatal centres, [6–8, 13, 15] while others were retrospectively conducted via the so-called 'cleft palate teams'. [14, 16] Consequently, the retrospective cleft team studies did not include the fetuses that were not born alive and were thus not referred to the cleft palate teams, thereby inducing selection bias. Additionally, according to Wyszynski et al,[17] information obtained from vital records (eg, birth certificates) is neither complete nor accurate in detail due to passive ascertainment methods (ie, data submitted by data sources and not actively collected by registry staff searching data sources for eligible cases) and lack of follow-up. Conversely, studies having active ascertainment methods or long follow-up periods, such as that of Rozendaal et al (median follow-up 5 years), [22] may result in relatively high rates of associated anomalies. Also, the value of information

depends on the interest and skills of the person who records the anomalies. This is in line with the study of Tan et al., [31] who reported higher frequencies of associated anomalies in patients recruited for a clinical study than in cases derived from a birth defect register. They suggested this was explained by a combination of ascertainment bias and more complete diagnosis by detailed clinical assessment in the clinical study.

Nevertheless, despite the above mentioned issues, we found unambiguous evidence that the three cleft categories are differently associated with structural and chromosomal defects. Due to the inclusion of large numbers of cases from both prenatal and postnatal populations, we were able to provide a rather reliable basis for clinicians and future parents, thereby allowing accurate counselling and informed decisions on whether to have invasive diagnostics if an oral cleft is detected prenatally.

Prenatal counselling and genetic testing

When counselling future parents regarding prognosis and risk of associated chromosomal defects, it is vital to tailor the discussion according to cleft category. As our results showed, CLP and CP are more frequently associated with additional anomalies and chromosomal defects than CL. Moreover, these frequencies are higher in bilateral than in unilateral CLP or CL±P. This emphasises the need for accurate prenatal subdivision of clefts. However, accurate detection of additional anomalies appears to be even more significant to outcome. As we found, the presence of other congenital anomalies is a strong predictor for chromosomal defects. For all cleft categories, both prenatal and postnatal studies showed that chromosomal abnormalities are almost always seen in association with other congenital anomalies. Therefore, invasive prenatal testing to identify chromosomal abnormalities in combination with genetic counselling should be offered in all cases with associated anomalies, irrespective of cleft category.

It should be realised, however, that the absence of associated anomalies does not exclude the possibility of the presence of an underlying chromosomal defect. As mentioned above, chromosomal analysis was often not performed in isolated cases, and therefore undetected chromosomal defects might have been included in our rates of isolated clefts. The few studies that reported chromosomal defects in isolated clefts showed that the prevalence differed by category. As standard karyotyping did not reveal any chromosomal defect, cases with isolated CL have the most favourable prognosis when it comes to chromosomal anomalies with a poor outcome. Therefore, if confident in ultrasound findings, conventional karyotyping is not recommended in isolated CL.

However, based on the findings of Rittler et al, [27] array CGH to detect deletion 22q11.2 should be considered.

For CLP, prenatal studies together showed chromosomal defects in 3.9% of the presumed isolated cases, while just one postnatal study addressed this issue showing defects in 1.0%. In the latter study, [27] standard karyotyping revealed chromosomal defects in 0.7% of the isolated CLP cases, while array CGH during follow-up revealed a deletion 22q11.2 for one more case. Based on these data, it is recommended to inform future parents about the possible association of a chromosomal defect and to consider invasive prenatal testing in these cases, preferably by array based methods. However, if not confident in ultrasound findings regarding cleft category, it should be noted that the overall prevalence in presumed isolated clefts (CP excluded) was 0.8% (7/830). Furthermore, when considering invasive testing, the baseline risk of complications (1%) should be weighed against the potential benefits. [42] Another concern might be the detection of unexpected or unclassified variants with array based methods, which should be discussed with future parents.

Regarding CP, especially isolated CP, prenatal identification is still challenging, which has resulted in limited prenatal information on their underlying chromosomal defects. However, postnatal karyotyping of isolated CP cases revealed a chromosomal defect in 1.6%. In this category in particular, specific syndromes, such as VCF (22q11.2 deletion), Treacher-Collins, and Stickler, have to be considered. As presented in supplementary table 2.2, these syndrome diagnoses were frequently reported in the evaluated literature. Therefore, until more information on chromosomal defects in prenatally presumed isolated CP is available, we advise consideration of invasive genetic testing and consultation by a clinical geneticist if an isolated CP is detected prenatally. A prenatal diagnostic algorithm according to cleft category is presented in Figure 2.1.

Figure 2.1

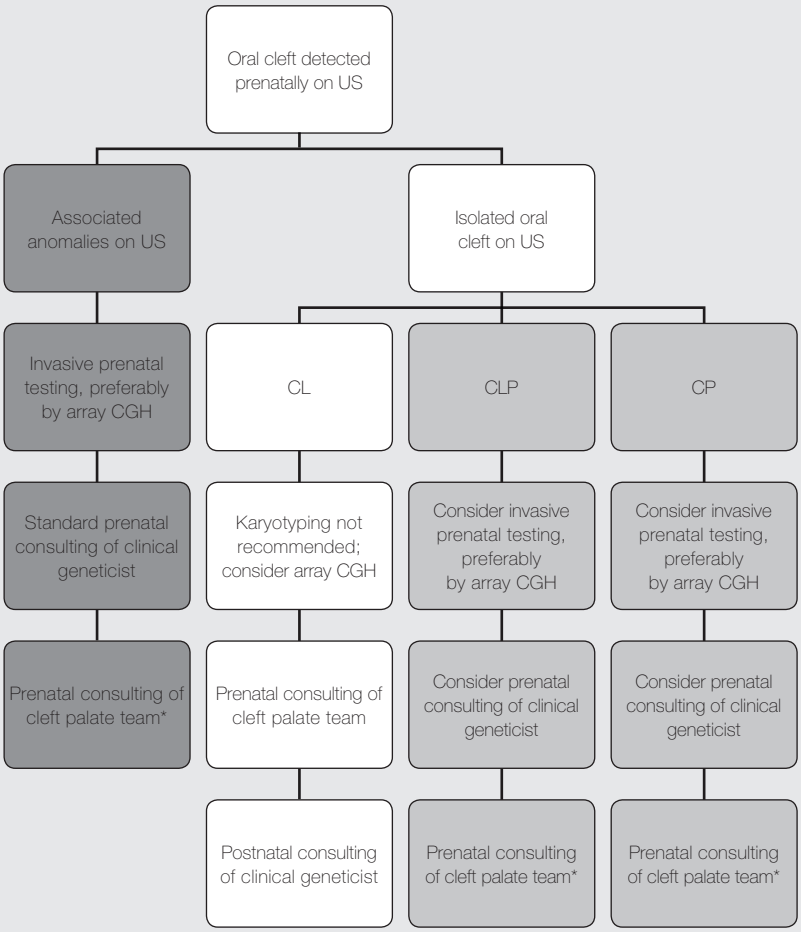


Figure 2.1 Algorithm for invasive genetic testing according to oral cleft category. array CGH, array comparative genomic hybridisation; CL, cleft lip only; CLP, cleft lip with cleft palate; CP, cleft palate only; US, ultrasound. *If a normal karyotype is confirmed or invasive genetic testing is declined.

Based on the above findings, more accurate prenatal ultrasound screening will improve counselling, especially regarding palatal involvement. Therefore, we advise the referral of pregnant women with a fetus suspected of having an oral cleft to a tertiary care centre

where more specific ultrasound screening can be performed. In addition, if a normal karyotype is confirmed or invasive testing is declined, future parents should be counselled by a multidisciplinary cleft palate team that focuses on psychosocial support, education on management of clefts, and parents' options, TOP being one of them. [43–46] Finally, it is crucial to distinguish median clefts and atypical facial clefts from oral clefts. These different craniofacial anomalies are associated with other congenital anomalies and have a different prognosis, and should therefore be referred to and treated by specialised multidisciplinary craniofacial teams.

Future studies

The use of array CGH in clinical practice is rising, and it is expected that it will be implemented as standard prenatal diagnostics in the near future. Compared to conventional karyotyping, array CGH can detect smaller chromosome deletions and duplications. To gain more insight into the yield of array CGH in cases with clefts, it would be interesting to perform array CGH in a large cohort of cases with prenatally and postnatally detected clefts. This would also give us more information about the proportion and types of chromosomal defects that are missed in cases that have not been karyotyped or studied by array based methods. Particularly with regard to prenatally presumed isolated clefts, this is essential to reach consensus on the role of invasive genetic testing in these cases. As was demonstrated by the NVSCA data, [23, 24] clefts can be associated with various microdeletions and duplications. This implies that array CGH should be the standard technique to identify chromosomal defects in children with oral clefts.

Finally, follow-up studies are needed to gain more insight into additional abnormalities and chromosomal anomalies identified after birth. This can aid in more optimal counselling of future parents, especially with regard to unexpected anomalies in presumed isolated clefts, and timely treatment of children with clefts.

Conclusions

This systematic review presents unambiguous evidence that the different cleft categories are variously associated with additional congenital anomalies and underlying chromosomal defects. This emphasises the need for accurate subdivision of CL, CLP and CP for both ultrasound screening and postnatal follow-up. However, the most important predictor of chromosomal abnormalities is the presence of associated anomalies, and we urge clinicians to advise invasive testing in these cases. In the absence of associated anomalies, cases with CL have the most favourable prognosis and do not require conventional karyotyping. In presumed isolated CLP and CP, professionals should explain the possible association of a chromosomal defect and consider invasive genetic testing, preferably by array based methods. In all cleft categories, an association with deletion 22q11.2 should be considered.

Accurate prenatal diagnosis by ultrasound is essential in the quality of counselling, especially with regard to palatal involvement and associated anomalies. Therefore, a pregnant woman with a fetus suspected of having an oral cleft should be referred to a tertiary care centre where more specific ultrasound screening can be performed. Finally, follow-up studies, including array CGH, are needed to gain more insight into additional abnormalities and chromosomal defects missed in associated and presumed isolated clefts. This would aid in more optimal counselling and timely treatment of children with oral clefts.

Acknowledgements

The authors would like to thank the clinical cytogeneticist Ellen van Binsbergen for her cytogenetic input as well as the clinical cytogeneticist Heleen Schuring-Blom and psychologist Henriëtte Swanenburg de Veye for their helpful discussion and critically reading of the manuscript. They would also like to express their gratitude to the Dutch cleft palate teams and the NVSCA, including its board. Without their efforts, the NVSCA would not have succeeded.

Supplementary table 2.1 Summary of the various study characteristics and designs of the prenatal and postnatal studies included in the systematic review

Study type	Study area	Study population and setting	Inclusion criteria	Data sources and collection
Prenatal studies				
Nyberg 1995	Not given	High-risk obstetric cases referred to tertiary referral centres 1988-1993*	Live births, stillbirths, and TOP with oral clefts (n=40)†	Prospective and retrospective analysis of sonograms & postnatal information retrieved by inspection at autopsy or after birth
Berge 2001	Region of Bonn, Germany	High-risk obstetric cases referred to a single tertiary referral centre 1991-2000*	Live births, stillbirths, and TOP with oral clefts (n=59)†	Prospective and retrospective analysis of sonograms & postnatal follow-up data from neonatologist and surgeons, and pathological and cytogenetic records
Perrotin 2001	Region of Tours, France	High-risk obstetric cases referred to a single tertiary referral centre 1991-1999*	Live births, stillbirths, and TOP with oral clefts (n=56)†	Retrospective evaluation of ultrasonographic and clinical records, and ascertainment via autopsy reports or hospital records with a postnatal follow-up of 3-12 months
Prenatal and Postnatal studies				
Offerdal 2008	Region of Trondheim, Norway	Pregnant women in a non-selected population who underwent routine ultrasound examination 1987-2004 (n=49,314)	Live births, stillbirths, and TOP (≥16 weeks gestation) with oral clefts (n=101)	Prospective collection of ultrasound examinations & prenatal and postnatal follow-up data from autopsy reports, photographs, physical examinations by paediatricians and review of medical records
Russell 2008	Novia Scotia, Canada	Births to residents of Novia Scotia registered in the Perinatal Database, Fetal Anomaly Database, or Cleft Palate Database 1992-2002 (n=108,220)	Live births, stillbirths, and TOP with oral clefts (n=225)	Data from the population-based Novia Scotia Atlee Perinatal Database, the Fetal Anomaly Database, and Cleft Palate Database
Gillham 2009	North-West England	Obstetric cases prenatally suspected of an oral cleft and referred to the Regional Fetal Management Unit & infants referred to the regional cleft team 2000-2006 (n=570)	Live births, stillbirths, and TOP with oral clefts (n=490)†	Retrospective review of prenatal diagnoses in the North-West Regional Fetal Management Unit Database & postnatal findings in the North-West Cleft Lip and Palate Database
Maarse 2011	Region of Utrecht, Netherlands	Pregnant women in a non-selected population who underwent routine ultrasound examination 2007-2008, including low-risk (n=35,924) and high-risk cases (n=2,836)	Live births, stillbirths, and TOP with oral clefts (n=60)†	Retrospective evaluation of ultrasound examinations from prenatal screening centres and clinical records of the cleft palate team

Table 2.1

Definition & classification associated congenital anomalies	Karyotype information
Major and minor congenital anomalies; IUGR and other ultrasound markers‡	Karyotype given for most associated clefts (94%, 17/18); number of isolated clefts karyotyped not given
Major and minor congenital anomalies; IUGR and other ultrasound markers‡	Karyotype given for most associated clefts (94%, 33/35) and isolated clefts (76%, 16/21)
Major and minor congenital anomalies; IUGR and other ultrasound markers	Karyotype given for all associated clefts (n=20), and some isolated clefts (39%, 14/36)
Structural congenital anomalies, sequences, and non-chromosomal syndromes according to the ICD; Additional anomalies further subdivided into: - chromosome aberrations - syndromes/sequences with normal chromosomes - structural anomalies without chromosomal aberrations, syndromes or sequences	Some clefts (31%, 31/101 were karyotyped; presence/absence of abnormal karyotype given for most associated clefts 62%, 29/47); no specific data given
Structural congenital anomalies; Additional anomalies further subdivided into: - with abnormal karyotype - structural/syndromic with normal karyotype	Data suggest that all associated clefts (n=99) were karyotyped; no specific data given
Structural congenital anomalies	Almost all associated clefts (97%, 29/30) were karyotyped; all isolated clefts were karyotyped
Major and minor congenital anomalies; IUGR and other ultrasound markers (20 weeks gestation); abnormal karyotype defined as associated anomaly	Some clefts were karyotyped; total number of associated and isolated clefts karyotyped not given

Postnatal studies				
Druschel 1996	New York State, USA	Children born to New York residents, registered with an oral cleft before the age of 2 years in the Congenital Malformation Registry, and matched to their birth certificate 1983-1990 (n=2,786)	Live births with oral clefts (n=2,786)	Retrospective review of data from the population-based database of New York State Congenital Malformations Registry and from additional registry sources, including birth certificates
Kallen 1996	Central-East of France/ Sweden/ California, USA	Births registered to 3 registries of the International Clearinghouse for Birth Monitoring Systems: France, 1976-1992 (n= 1.46 million); Sweden, 1973-1992 (n=2.07 million); California, 1983-1990 (n=1.62 million)	Live births and stillbirths (≥28 weeks gestation) with oral clefts (n=8,315)	Data from the population-based databases of Central-East France, National Sweden's Registry, and the California Birth Defects Monitoring Program; malformations were retrospectively ascertained up to an age of one year
Milerad 1997	Region of Stockholm, Sweden	Children with oral clefts referred to the regional cleft palate team of Stockholm or reported to the National Malformation Registry 1975-1992 (n=616)	Live births with oral clefts (n=616; submucous CP excluded)	Retrospective review of cleft palate team files, and birth and hospital records
Walker 2001	Utah, USA	Births to residents of the statewide non-selected non-referred population of Utah 1995-1999 (n=217,429)	Live births, stillbirths, and TOP with CL or CLP (n=263; CP only excluded)	Data from the Utah Department of Health Birth Defect Network population-based surveillance system
DeRoo 2003	Washington State, USA	Live-born infants to residents of the statewide population of Washington 1987-1990 (n=298,138)	Live births with oral clefts (n=608)	Data from the Washington State Birth Defects Registry population-based surveillance system on congenital anomalies diagnosed within the first year of life & Washington State birth certificates
Shaw 2004	California, USA	Deliveries (≥20 weeks gestation) to California women in non-military hospitals 1983-1997 (n=3,572,230)	Live births, stillbirths, and TOP (≥20 weeks gestation) with oral clefts (n=6,415)	Data from the California Birth Defects Monitoring Program, population-based active surveillance system on congenital anomalies diagnosed within 1 year of delivery
Vallino-Napoli 2006	Victoria, Australia	Pregnancies in Victoria 1983-2000 (n=1,140,668)	Live births, stillbirths, and TOP (<20 weeks gestation) with oral clefts (n=2,022)	Data from the population-based Victorian Birth Defects Registry

Table 2.1

Major congenital anomalies, and minor congenital anomalies (only if they were associated with major anomalies)	No information given
Major non-facial congenital structural anomalies	Identified karyotypes presented; number of associated and isolated clefts karyotyped not given
Congenital anomalies that require follow-up or intervention; Abnormal karyotype defined as associated anomaly	Most identified karyotypes (94%, 16/17) presented; not classified according cleft category
Major anatomic congenital anomalies (that is, those anomalies that would alter pregnancy management or result in functional impairment of the child)	Most associated clefts (75%, 38/51), and few isolated clefts (0.6%, 12/212) were karyotyped
Major congenital anomalies, confirmed genetic anomalies and recognized syndromes	No information given
Structural congenital anomalies according to the BPA; Abnormal karyotype defined as associated anomaly	No information given
Major congenital anomalies, PRS, chromosomal anomalies, and non-chromosomal syndromes	Some clefts karyotyped; number of associated and isolated clefts karyotyped not given

Postnatal studies				
Calzolari 2007	Europe	Births registered to 23 registers in 14 European countries 1980-2000 (n=5,989,834)	Live births, stillbirths, and TOP with CL or CLP (n=5,449)	Data from the European network (EUROCAT) of 23 registers in 14 European countries, having various periods of follow-up
Stoll 2007	Region of Strasbourg, France	Newborns and foetuses delivered in 11 maternity hospitals in Strasbourg and surrounding rural areas 1979-2003 (n=334,262; no home deliveries in this area)	Live births, stillbirths, and TOP with oral clefts (n=651; submucous CP excluded)	Data from the regional registry of congenital malformation on anomalies diagnosed within 1 year of age
Beriaghi 2009	Omaha, Nebraska, USA	Children with oral clefts referred to the cleft palate/craniofacial clinic 1980-2000 (n=1,127)	Live births with oral clefts (n=1,127)	Data from the craniofacial centre database obtained by the multidisciplinary team
Tan 2009	Victoria, Australia	Children born in Victoria and registered with an oral cleft in the Birth Defects Register 2000-2002 (n=312)	Live births with oral clefts (n=279)‖	Data from the Victorian Birth Defects Register on congenital anomalies diagnosed within 15 years of age
Rittler 2011	South America	Children with congenital anomalies ascertained at birth in 48 maternity hospitals from 7 countries of the ECLAMC network, within the framework of a special intervention study 2003-2005 (n=10,371)¶	Live births with oral clefts (n=710; those with a bifid uvula, congenitally 'healed' CL, submucous CP, or birth weight < 500 g excluded)	Information reported by paediatricians and retrieved by further evaluation by dysmorphologists and geneticists during a follow-up period of 1 year
Rozendaal 2011	Netherlands	Children with oral clefts referred to the 15 Dutch cleft palate teams in the Netherlands	Live births with oral clefts	Registry data from the National Oral Cleft Database and review of medical records (including colour photographs, X-rays, and dental casts) after a median follow-up period of 5 years

TOP, termination of pregnancy; CL, cleft lip only; CLP, cleft lip with cleft palate; CP, cleft palate only; IUGR, intrauterine growth retardation; MCA, multiple congenital anomalies; PRS, Pierre Robin Sequence; EUROCAT, European Registry of Congenital Anomalies and Twins; ECLAM, Latin American Collaborative Study of Congenital Malformations; ICD, International Classification of Diseases (9th and 10th revision); BPA, malformation codes of the British Pediatric Association.
* Number of cases not given.
† Median cleft lip and/or atypical facial clefts analyzed, but excluded from this review.

Table 2.1

Two or more unrelated congenital anomalies according to the BPA; Additional anomalies further subdivided into: - recognized conditions (including chromosomal) - MCA	Some clefts were karyotyped; number of associated and isolated clefts karyotyped not given
One or more non-cleft major congenital anomalies§; Additional anomalies further subdivided into: - chromosomal - non-chromosomal	All associated clefts were karyotyped
Congenital anomalies (slight variations of normal & neurological and behavioural abnormalities excluded); Abnormal karyotype defined as associated anomaly	No information given
Structural, functional, genetic, chromosomal, and biochemical abnormalities after birth	Some clefts were karyotyped; number of associated and isolated clefts karyotyped not given
Major unrelated defects (that is, those requiring medical or surgical intervention, or of substantial cosmetic importance, and clinically recognizable or suspected syndromes) detected and reported between birth and hospital discharge; PRS was classified as isolated CP; Additional anomalies further subdivided into: - chromosomal anomaly - syndromes without chromosomal anomalies - malformation complexes/sequences - MCA	All clefts were karyotyped; information available for most associated (58%, 108/185) and isolated clefts (54%, 281/525); FISH 22q11 was not regularly performed; array CGH was performed in some clefts
Major and minor congenital anomalies, including dysmorphic features	Some associated and isolated clefts karyotyped; number not given

‡ Other ultrasound markers: e.g., oligohydramnios, polyhdyramnios, single umbilical artery, or nuchal oedema.
§ Stoll et al. excluded mental retardation and classified Pierre Robin sequence as isolated CP when it was present without congenital anomalies beyond micrognathia and glossoptosis.
|| Cases with PRS (n=33) analyzed, but excluded from this review because of the inconsistent definition applied in clinical practice and consequently its over- or underreporting.
¶ Argentina, Bolivia, Brazil, Chile, Colombia, Ecuador, and Venezuela (total live births in this area: n=422,240).

Table 2.2

Supplementary table 2.2 Observed chromosomal defects and non-chromosomal syndromes associated with oral clefts in prenatal and/or postnatal populations

	Studies												
	Prenatal			Prenatal-Postnatal			Postnatal						
	Nyberg 1995	Berge 2001	Perrotin 2001	Maarse 2010	Russell 2008	Gillham 2009	Kallen 1996	Millerad 1997	Stoll 2007	Vallino 2006	Walker 2001	Rittler 2011	NWSCA 1997-2010*
Chromosomal defect													
Trisomy 6											X		
Trisomy 9p				X									
Trisomy 13	X	X	X	X	X	X		X	X	X	X	X	X
Trisomy 16p													X
Trisomy 18	X	X	X	X	X	X		X	X	X	X	X	X
Trisomy 21				X				X	X	X		X	X
Trisomy 22								X					
Triploidy 69		X											
Monosomy 21								X					
Mosaic trisomy 22	X												
Mosaic tetrasomy 12p												X	
Partial autosomal trisomy	X								X				
Translocation			X		X								
Deletion 2q								X					
Deletion 4p			X									X	X
Deletion 4q								X					
Deletion 5p14.3p14.1													X
Deletion 5q21.1q23.3													X
Deletion 13q											X		X
Deletion 22q11.2									X			X	X
46,XY,der(3)del(p26)inv dup(3)(p24p25)													X
46,XX, der(6)t(2;6)(q37;q27)pat													X
dup(11)(p11.1p15.5)pat													X
46,XY,der(14)t(14;16)(p11;p12.3)													X
46,XY,add(15)(p11)												X	
46,XX,del(16)(q22.3q22.3)													X
46,XY,der(18)t(16;18)(q24;q23)pat													X

Supplementary table 2.2 Observed chromosomal defects and non-chromosomal syndromes associated with oral clefts in prenatal and/or postnatal populations

Table 2.2

	Studies												
	Prenatal			Prenatal-Postnatal			Postnatal						
	Nyberg 1995	Berge 2001	Perrotin 2001	Maarse 2010	Russell 2008	Gilham 2009	Kallen 1996	Mlierad 1997	Stoll 2007	Vallino 2006	Walker 2001	Rittler 2011	NVSCA 1997-2010*
Chromosomal defect													
46,XY,del(18)(q21.3)													X
47,XX,+inv dup (22)(q11q11)													X
dup(22)(q11q11)													X
Partial autosomal deletion	X								X				X
Sex chromosomal abnormalities	X							X	X			X	X
Other chromosomal abnormalities		X								X			
Non-chromosomal syndrome													
Adams-Oliver syndrome									X				
Amniotic band association	X									X			
Anti-epileptic drugs							X						
Apert syndrome						X	X	X					X
Beckwith-Wiedeman syndrome				X				X					X
Branchio-oculo-facial syndrome													X
Bohring-Opitz syndrome													X
Caudal Regression syndrome					X								
CHARGE syndrome						X						X	X
Chondrodystrophy							X						
Cornelia de Lange syndrome							X		X			X	X
Crouzon syndrome								X					X
DiGeorge syndrome					X					X			
Duane retraction syndrome													X
Ectrodactyly-ectodermal dysplasia-clefting syndrome							X		X				X
Encephalocele-clefting syndrome								X					
Foetal alcohol syndrome							X						
Fraser syndrome													X
Fryns syndrome			X										
Goldenhar syndrome				X			X		X	X			X

Table 2.2

Supplementary table 2.2 Observed chromosomal defects and non-chromosomal syndromes associated with oral clefts in prenatal and/or postnatal populations

	Studies												
	Prenatal			Prenatal-Postnatal			Postnatal						
	Nyberg 1995	Berge 2001	Perrotin 2001	Maarse 2010	Russell 2008	Gillham 2009	Kallen 1996	Milerad 1997	Stoll 2007	Vallino 2006	Walker 2001	Rittler 2011	NVSCA 1997-2010*
Gordon syndrome													X
Gorlin syndrome													X
Greig syndrome													X
Hay-Wells (AEC) syndrome													X
Holoprosencephaly										X			
Ivemark syndrome									X				
Jeune syndrome													X
Kabuki syndrome													
Kartagener syndrome†								X					
Klippel-Feil syndrome							X		X				X
Larsen syndrome								X				X	
Loeys-Dietz syndrome													X
Meckel-Gruber syndrome					X								
Meckel syndrome							X		X				
Moebius syndrome							X		X				X
Mohr syndrome												X	
Multiple epifysial dysplasia†										X			
Multiple pterygium syndrome									X				
Nager syndrome							X						X
Noonan syndrome													X
Omenn reticuloendotheliosis†									X				
Opitz-Frias syndrome			X										
Opitz G/BBB												X	X
Oro-facio-digital syndrome							X		X				X
Osler-Weber syndrome†								X					
Osteogenesis imperfecta†							X						
Osteopathia striata with cranial sclerosis													X
Oto-palato-digital syndrome	X								X				X

Supplementary table 2.2 Observed chromosomal defects and non-chromosomal syndromes associated with oral clefts in prenatal and/or postnatal populations

Table 2.2

	Studies												
	Prenatal			Prenatal-Postnatal			Postnatal						
	Nyberg 1995	Berge 2001	Perrotin 2001	Maarse 2010	Russell 2008	Gilham 2009	Kallen 1996	Mlierad 1997	Stoll 2007	Valino 2006	Walker 2001	Rittler 2011	NVSCA 1997-2010*
Pentalogy of Cantrell													
(Thoraco-abdominal syndrome)													X
Poland syndrome†								X					
Popliteal pterygium syndrome			X				X						
Rieger syndrome													X
Roberts syndrome									X				
Robinow syndrome								X					
Rubinstein-Taybi syndrome†											X	X	X
Smith-Lemli-Opitz syndrome					X		X	X					X
Stickler syndrome				X		X	X	X	X			X	X
Treacher-Collins syndrome							X	X	X	X			X
VACTERL													X
Van der Woude syndrome				X	X			X			X	X	X
VATER association									X				
VCF syndrome§									X				
X-linked hydrocephalus†										X			
Other diagnosis													
Neonatal Abstinence syndrome													X
Pierre Robin sequence				X	X	X			X	X			X
Sebaceus Nevus syndrome													X

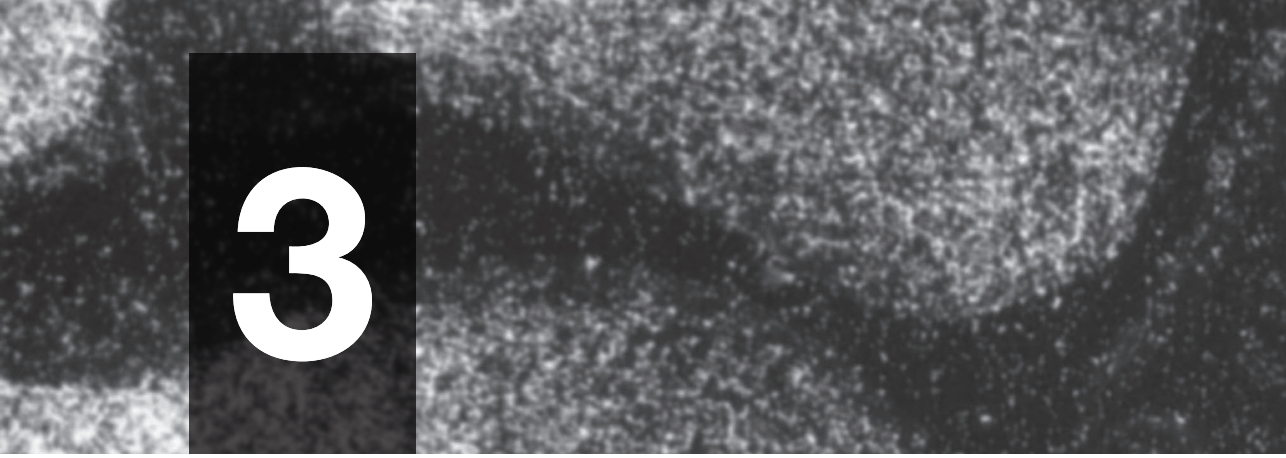
* Annual reports 1997-2010 of the Dutch Association for Cleft Palate and Craniofacial anomalies, comprising data without follow-up.
† Diagnosis uncertain.
§ Clinical diagnosis, not confirmed by karyotype.

References

1. Mossey PA, Little J, Munger RG, Dixon MJ, Shaw WC. Cleft lip and palate. Lancet 2009;374:1773–85.
2. Luijsterburg AJM, Vermeij-Keers C. Recording and classification common oral clefts. In: Lilja J Transactions, the 9th international congress on cleft palate and related craniofacial anomalies. Götenborg: Elanders Novum, 2001:243–50.
3. Harville EW, Wilcox AJ, Lie RT, Vindenes H, Abyholm F. Cleft lip and palate versus cleft lip only: are they distinct defects? Am J Epidemiol 2005;162:448–53.
4. Rittler M, Lopez-Camelo JS, Castilla EE, Bermejo E, Cocchi G, Correa A, Csaky-Szunyogh M, Danderfer R, De Vigan C, De Walle H, Da Graca Dutra M, Hirahara F, Martinez-Frias ML, Merlob P, Mutchinick O, Ritvanen A, Robert-Gnansia E, Scarano G, Siffel C, Stoll C, Mastroiacovo P. Preferential associations between oral clefts and other major congenital anomalies. Cleft Palate Craniofac J 2008;45:525–32.
5. Luijsterburg AJM, Vermeij-Keers C. Ten years recording common oral clefts with a new descriptive system. Cleft Palate Craniofac J 2011;48:173–82.
6. Nyberg DA, Sickler GK, Hegge FN, Kramer DJ, Kropp RJ. Fetal cleft lip with and without cleft palate: US classification and correlation with outcome. Radiology 1995;195:677–84.
7. Berge SJ, Plath H, Van de Vondel PT, Appel T, Niederhagen B, Von Lindern JJ, Reich RH, Hansmann M. Fetal cleft lip and palate: sonographic diagnosis, chromosomal abnormalities, associated anomalies and postnatal outcome in 70 fetuses. Ultrasound Obstet Gynecol 2001;18:422–31.
8. Perrotin F, De Poncheville LM, Marret H, Paillet C, Lansac J, Body G. Chromosomal defects and associated malformations in fetal cleft lip with or without cleft palate. Eur J Obstet Gynecol Reprod Biol 2001;99:19–24.
9. Van der Meulen J, Mazzola R, Strickler M, Raphael B. Classification of craniofacial malformations. In: Stricker M, Van der Meulen JC, Raphael B, Mazzola R, Tolhurst DE, Murray JE Craniofacial malformations. Edinburgh: Churchill Livingstone, 1990:149–309.
10. Vermeij-Keers C. Craniofacial embryology and morphogenesis: normal and abnormal. In: Stricker M, Van der Meulen JC, Raphael B, Mazzola R, Tolhurst DE, Murray JE Craniofacial malformations. Edinburgh: Churchill Livingstone, 1990:27–60.
11. Maarse W, Berge SJ, Pistorius L, Van Barneveld T, Kon M, Breugem C, Van der Molen AB. Diagnostic accuracy of transabdominal ultrasound in detecting prenatal cleft lip and palate: a systematic review. Ultrasound Obstet Gynecol 2010;35:495–502.
12. Stoll C, Dott B, Alembik Y, Roth M. Evaluation of prenatal diagnosis of cleft lip/palate by foetal ultrasonographic examination. Ann Genet 2000;43:11–14.
13. Maarse W, Pistorius LR, Van Eeten WK, Breugem CC, Kon M, Van den Boogaard MJ, Mink van Der Molen AB. Prenatal ultrasound screening for orofacial clefts. Ultrasound Obstet Gynecol 2011;38:434–9.
14. Gillham JC, Anand S, Bullen PJ. Antenatal detection of cleft lip with or without cleft palate: incidence of associated chromosomal and structural anomalies. Ultrasound Obstet Gynecol 2009;34:410–15. [CrossRef][Medline]
15. Offerdal K, Jebens N, Syvertsen T, Blaas HG, Johansen OJ, Eik-Nes SH. Prenatal ultrasound detection of facial clefts: a prospective study of 49,314 deliveries in a non-selected population in Norway. Ultrasound Obstet Gynecol 2008;31:639–46.

16. Russell KA, Allen VM, MacDonald ME, Smith K, Dodds L. A population-based evaluation of antenatal diagnosis of orofacial clefts. Cleft Palate Craniofac J 2008;45:148–53.
17. Wyszynski DF, Sarkozi A, Czeizel AE. Oral clefts with associated anomalies: methodological issues. Cleft Palate Craniofac J 2006;43:1–6.
18. Altherr MR, Bengtsson U, Elder FF, Ledbetter DH, Wasmuth JJ, McDonald ME, Gusella JF, Greenberg F. Molecular confirmation of Wolf-Hirschhorn syndrome with a subtle translocation of chromosome 4. Am J Hum Genet 1991;49:1235–42.
19. Desmaze C, Scambler P, Prieur M, Halford S, Sidi D, Le Deist F, Aurias A. Routine diagnosis of DiGeorge syndrome by fluorescent in situ hybridization. Hum Genet 1993;90:663–5.
20. Rozendaal AM, Luijsterburg AJM, Mohangoo AD, Ongkosuwito EM, Anthony S, Vermeij-Keers C. Validation of the NVSCA registry for common oral clefts: study design and first results. Cleft Palate Craniofac J 2010;47:534–43.
21. Rozendaal AM, Luijsterburg AJM, Mohangoo AD, Ongkosuwito EM, De Vries E, Vermeij-Keers C. Validation of the Dutch Registry of Common Oral Clefts: quality of recording specific oral cleft features. Cleft Palate Craniofac J. Published Online First: 23 November 2010. doi:10.1597/10-109.
22. Rozendaal AM, Luijsterburg AJM, Ongkosuwito EM, Van den Boogaard M-JH, De Vries E, Hovius SER, Vermeij-Keers C. Delayed diagnosis and underreporting of congenital anomalies associated with oral clefts in the Netherlands: a national validation study. J Plast Reconstr Aesthet Surg 2012;65:780–90.
23. Luijsterburg AJM, Vermeij-Keers C. NVSCA registry common oral clefts. Annual reports 1997–2007 of the Dutch Association for Cleft Palate and Craniofacial Anomalies (in Dutch). Rotterdam: Department of Plastic and Reconstructive Surgery, Erasmus MC, University Medical Center Rotterdam, 1999–2008. ISSN 1571-876X.
24. Luijsterburg AJM, Rozendaal AM, Trenning AH, Ongkosuwito EM, Vermeij-Keers C. NVSCA registry common oral clefts. Annual reports 2008–2010 of the Dutch Association for Cleft Palate and Craniofacial Anomalies (in Dutch). Rotterdam: Department of Plastic and Reconstructive Surgery, Erasmus MC, University Medical Center Rotterdam, 2010–2011. ISSN 1571-876X.
25. Walker SJ, Ball RH, Babcook CJ, Feldkamp MM. Prevalence of aneuploidy and additional anatomic abnormalities in fetuses and neonates with cleft lip with or without cleft palate: a population-based study in Utah. J Ultrasound Med 2001;20:1175–80; quiz 81-2.
26. Stoll C, Alembik Y, Dott B, Roth MP. Associated malformations in patients with oral clefts. Am J Med Genet A 2007;143A:2463–5.
27. Rittler M, Cosentino V, Lopez-Camelo JS, Murray JC, Wehby G, Castilla EE. Associated anomalies among infants with oral clefts at birth and during a 1-year follow-up. Am J Med Genet A 2011;155A:1588–96.
28. Kallen B, Harris J, Robert E. The epidemiology of orofacial clefts. 2. Associated malformations. J Craniofac Genet Dev Biol 1996;16:242–8.
29. Vallino-Napoli LD, Riley MM, Halliday JL. An epidemiologic study of orofacial clefts with other birth defects in Victoria, Australia. Cleft Palate Craniofac J 2006;43:571–6.
30. Calzolari E, Pierini A, Astolfi G, Bianchi F, Neville AJ, Rivieri F. Associated anomalies in multi-malformed infants with cleft lip and palate: an epidemiologic study of nearly 6 million births in 23 EUROCAT registries. Am J Med Genet A 2007;143:528–37.
31. Tan TY, Amor DJ, Riley M, Halliday J, Kilpatrick N, Simms K, White SM. Registry- and clinic-based analyses of birth defects and syndromes associated with cleft lip/palate in Victoria, Australia. Cleft Palate Craniofac J 2009;46:583–7.

32. Druschel CM, Hughes JP, Olsen CL. First year-of-life mortality among infants with oral clefts: New York State, 1983–1990. Cleft Palate Craniofac J 1996;33:400–5.
33. Milerad J, Larson O, Ph DD, Hagberg C, Ideberg M. Associated malformations in infants with cleft lip and palate: a prospective, population-based study. Pediatrics 1997;100:180–6.
34. DeRoo LA, Gaudino JA, Edmonds LD. Orofacial cleft malformations: associations with maternal and infant characteristics in Washington State. Birth Defects Res A Clin Mol Teratol 2003;67:637–42.
35. Shaw GM, Carmichael SL, Yang W, Harris JA, Lammer EJ. Congenital malformations in births with orofacial clefts among 3.6 million California births, 1983–1997. Am J Med Genet A 2004;125A:250–6.
36. Beriaghi S, Myers SL, Jensen SA, Kaimal S, Chan CM, Schaefer GB. Cleft lip and palate: association with other congenital malformations. J Clin Pediatr Dent 2009;33:207–10.
37. Chmait R, Pretorius D, Moore T, Hull A, James G, Nelson T, Jones M. Prenatal detection of associated anomalies in fetuses diagnosed with cleft lip with or without cleft palate in utero. Ultrasound Obstet Gynecol 2006;27:173–6.
38. Swanenburg de Veye HF, Beemer FA, Mellenbergh GJ, Wolters WH, Heineman-de Boer JA. An investigation of the relationship between associated congenital malformations and the mental and psychomotor development of children with clefts. Cleft Palate Craniofac J 2003;40:297–303.
39. Timmerman E, Pajkrt E, Maas SM, Bilardo CM. Enlarged nuchal translucency in chromosomally normal fetuses: strong association with orofacial clefts. Ultrasound Obstet Gynecol 2010;36:427–32.
40. Wilhelm L, Borgers H. The 'equals sign': a novel marker in the diagnosis of fetal isolated cleft palate. Ultrasound Obstet Gynecol 2010;36:439–44.
41. Breugem CC, Mink van der Molen AB. What is 'Pierre Robin sequence'? J Plast Reconstr Aesthet Surg 2009;62:1555–8.
42. Tabor A, Philip J, Madsen M, Bang J, Obel EB, Norgaard-Pedersen B. Randomised controlled trial of genetic amniocentesis in 4606 low-risk women. Lancet 1986;1:1287–93.
43. Davalbhakta A, Hall PN. The impact of antenatal diagnosis on the effectiveness and timing of counselling for cleft lip and palate. Br J Plast Surg 2000;53:298–301.
44. Jones MC. Prenatal diagnosis of cleft lip and palate: detection rates, accuracy of ultrasonography, associated anomalies, and strategies for counseling. Cleft Palate Craniofac J 2002;39:169–73.
45. Byrnes AL, Berk NW, Cooper ME, Marazita ML. Parental evaluation of informing interviews for cleft lip and/or palate. Pediatrics 2003;112:308–13.
46. Johnson N, Sandy R. Prenatal diagnosis of cleft lip and palate. Cleft Palate Craniofac J 2003;40:186–9.



3

Chapter 3

MSX1 causes clefting in humans

MSX1 mutation is associated with orofacial clefting and tooth agenesis in humans.

van den Boogaard MJ, Dorland M, Beemer FA, Ploos van Amstel HK.

Published in: Nat Genet. 2000 Apr;24(4):342-3.

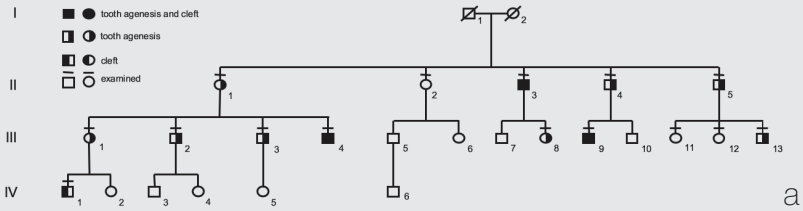
A Dutch family with tooth agenesis and various combinations of cleft palate only and cleft lip and cleft palate showed a nonsense mutation (p.Ser111*) in exon 1 of *MSX1*. The mutant phenotype of the family is similar to that of the *Msx1*-mutant mouse.

Nonsyndromic cleft lip with or without cleft palate (CL/P, OMIM 119530) and isolated cleft palate (CPO, OMIM 119540) are common malformations. The prevalence of orofacial clefts at birth varies from 1 in 500 to 1 in 2,500 and depends on ethnic background, geographic origin and socioeconomic status. [1, 2, 3, 4, 5] The aetiology of orofacial clefting is complex and both genetic and environmental factors are involved. [1] Genetic epidemiological studies suggest that several interacting loci, including a major gene, are involved in the aetiology of cleft palate, possibly accounting for approximately one-half of the familial occurrences. [6, 7] Several candidate genes for orofacial clefting, including *TGFA*, *BCL3*, *DLX2*, *MSX1* and *TGFB3*, have been screened for linkage-disequilibrium (LD) with CL/P or CPO. [8] Significant LD was found between CL/P and neutral polymorphisms within *MSX1* and *TGFB3* and between CPO and MSX1, respectively, suggesting involvement of these genes in orofacial clefting. A large panel of patients with CPO or CL/P (69 and 24, respectively) was screened for *MSX1* mutations, but no disease-specific abnormalities were found. [8]

Msx1-deficient mice show multiple craniofacial defects including clefting of the secondary palate, abnormalities of several facial bones and the malleus in the middle ear, and complete tooth agenesis. [9] These observations established *MSX1* as a candidate gene for involvement in both cleft palate and selective tooth agenesis in human. In this issue, *Msx2*-mutant mice [10] and *MSX2* haploinsufficiency in human [11] are reported. The mutations are associated with defects in skull ossification and with dental abnormalities. Clefting, however, was not observed.

So far, a single human *MSX1* missense mutation has been reported in a family with autosomal dominant selective tooth agenesis [12] (OMIM 106600); however, the affected individuals had no other craniofacial abnormalities. We investigated a Dutch family with 12 affected family members showing various combinations of cleft lip, cleft palate and tooth agenesis (Fig. 3.1a). We found four male family members to have a cleft: the five-year-old male proband (Fig. 3.1a, individual IV-1) and individual III-4 had a cleft palate, II-3 had a cleft alveolar ridge and III-9 had a cleft lip and palate.

Figure 3.1



Pedigree numbers (fig. 1a)		Left								Right							
		8	7	6	5	4	3	2	1	1	2	3	4	5	6	7	8
II-1	Mx.														•		
	Mn.			•					•	•							
II-4	Mx.	•			•									•			•
	Mn.	•			•									•			•
II-5	Mx.	•			•	•								•			•
	Mn.	•			•									•		•	•
III-1	Mx.	•			•	•							•	•			•
	Mn.	•			•									•			•
III-4	Mx.				•									•			
	Mn.				•									•			
III-8	Mx.				•	•		•			•		•	•			
	Mn.																
III-9	Mx.				•	•		•					•	•			
	Mn.	•			•									•			•
III-13	Mx.	•	•		•			•			•			•		•	•
	Mn.	•	•		•								•	•	•	•	•

b

Figure 3.1 Segregation of clefting and tooth agenesis. | a, Pedigree and symptoms of the Dutch family with clefting and tooth agenesis. | b, Confirmed dentition in affected family members (1, central incisor; 2, lateral incisor; 3, canine; 4, 5, first and second premolar; 6, 7, 8, first, second and third molar, respectively). Mx., maxilla; Mn., mandibula; • , missing teeth.

The variations in dentition status are given (Fig. 3.1b). Eleven family members lacked some permanent teeth. Three individuals (II-3, III-2 and III-3) were under medical care for missing teeth at the time of investigation and information about the identity and number of their missing teeth was not available. (Fig. 3.1b only includes verified tooth agenesis.) Most affected individuals (II-4, II-5, III-1, III-4, III-9 and III-13) were missing both mandibular and maxillary second premolars (Fig. 3.1b, position 5). The third molar at position 8 was also frequently absent (individuals II-4, II-5, III-1, III-9 and III-13). In general, most examples of dental agenesis were bilaterally symmetrical.

Direct sequencing of *MSX1* in individual IV-1 (Fig. 3.1a) using described primers [12] revealed a heterozygous C ->A transition at c.332 ([13]; Fig. 3.2 a). This creates a stop codon in exon 1 (p.Ser111*) and disrupts an *MboII* site, which was used to identify mutation carriers in the family (Fig. 3.2 b). We found the mutation to be heterozygous in all 12 affected family members (clefting and/or missing teeth), but not in the three unaffected. Further, the Ser111* mutation was not present in 102 control chromosomes.

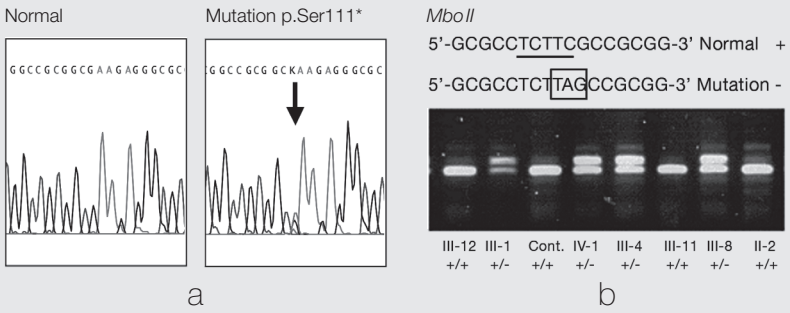


Figure 3.2

Figure 3.2 *MSX1* nonsense mutation. a. Reverse sequence of a part of exon 1 of *MSX1* showing the substitution producing the p.Ser111*. b. *MboII* digestion after PCR amplification with forward and reverse primers specific for exon 1 (ref. 12). The mutation causes loss of an *MboII* site, resulting in separation of a larger band following agarose electrophoresis.

The p.Arg202Pro MSX1 protein has a perturbed structure, a reduced thermostability, was inactive in vivo and did not antagonize the activity of wild-type protein [14]. It was concluded that haploinsufficiency was the probable cause for the mutant phenotype and not a dominant-negative affect, as originally proposed. [12] Our finding of a stop mutation in exon 1, proximal to the important Antp homeodomain in exon 2, is also likely to lead to haploinsufficiency.

The phenotype in the Dutch family presented here appears to be more severe than that described for the p.Arg202Pro missense mutation in *MSX1* (reported as a Arg31Pro substitution in exon 2 of *MSX1*), and apparently gives rise solely to tooth agenesis [12] in all 12 affected family members. [12] The pattern of tooth agenesis was almost identical in the two families. In the Dutch family, however, the presence of clefting variations in 4 of 12 affected individuals demonstrates the variability in penetrance and expressivity of this

feature. Previous studies suggested aetiological distinction between isolated cleft palate and cleft lip with or without cleft palate. [15] In the Dutch *MSX1* family, combinations of cleft palate only and cleft lip and cleft palate are present. This is consistent with the linkage disequilibrium observed for both types of clefting with *MSX1*. [8] It may be that the absence of clefting in the p.Arg202Pro family is a sampling affect and that availability of more affected individuals would have eventually revealed clefting defects in that family also. Again, differences in genetic background or environment might be responsible for clefting differences between the two families.

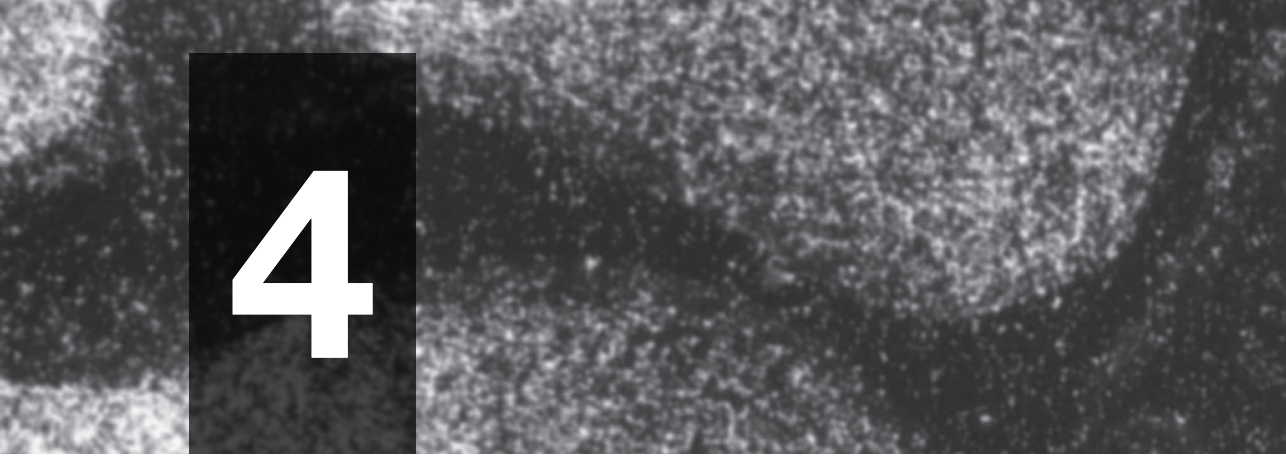
We conclude that the mutant phenotype of the family presented here has orofacial clefting similar to that of the *Msx1*-mutant mouse [9], in addition to the dental agenesis. [12] Although this confirms *MSX1* as a candidate gene for orofacial clefting, it is necessary to ascertain further human families exhibiting association between *MSX1* mutations and orofacial clefting and dental agenesis.

Acknowledgments

We thank P.L. Pearson for his suggestions in preparing this manuscript.

References

1. Schutte BC, Murray JC. The many faces and factors of orofacial clefts. Hum Mol Genet. 1999;8:1853-9.
2. Vanderas, AP. Incidence of cleft lip, cleft palate, and cleft lip and palate among races: a review. Cleft Palate J. 1987;24:216-25
3. Murray JC, Daack-Hirsch S, Buetow KH, Munger R, Espina L, Paglinawan N, Villanueva E, Rary J, Magee K, Magee W. Clinical and epidemiologic studies of cleft lip and palate in the Philippines. Cleft Palate Craniofac J. 1997;34:7-10.
4. Croen LA, Shaw GM, Wasserman CR, Tolarová MM. Racial and ethnic variations in the prevalence of orofacial clefts in California, 1983-1992. Am J Med Genet. 1998;79:42-7.
5. Tolarová MM, Cervenka J.Classification and birth prevalence of orofacial clefts. Am J Med Genet. 1998;75:126-37.
6. FitzPatrick D, Farrall M. J. An estimation of the number of susceptibility loci for isolated cleft palate. J Craniofac Genet Dev Biol. 1993;13:230-5.
7. Christensen K, Mitchell LE. Familial recurrence-pattern analysis of nonsyndromic isolated cleft palate--a Danish Registry study. Am J Hum Genet. 1996;58:182-90.
8. Lidral AC, Romitti PA, Basart AM, Doetschman T, Leysens NJ, Daack-Hirsch S, Semina EV, Johnson LR, Machida J, Burds A, Parnell TJ, Rubenstein JL, Murray JC. Association of MSX1 and TGFB3 with nonsyndromic clefting in humans. Am J Hum Genet. 1998;63:557-68.
9. Satokata I, Maas R. Msx1 deficient mice exhibit cleft palate and abnormalities of craniofacial and tooth development. Nat Genet. 1994;6:348-56.
10. Satokata I, Ma L, Ohshima H, Bei M, Woo I, Nishizawa K, Maeda T, Takano Y, Uchiyama M, Heaney S, Peters H, Tang Z, Maxson R, Maas R. Msx2 deficiency in mice causes pleiotropic defects in bone growth and ectodermal organ formation. Nat Genet. 2000;24:391-5.
11. Wilkie AO, Tang Z, Elanko N, Walsh S, Twigg SR, Hurst JA, Wall SA, Chrzanowska KH, Maxson RE Jr. Functional haploinsufficiency of the human homeobox gene MSX2 causes defects in skull ossification. Nat Genet. 2000;24:387-90.
12. Vastardis, H., Karimbux, N., Guthua, S.W., Seidman, J.G. & Seidman, C.E. A human MSX1 homeodomain missense mutation causes selective tooth agenesis. Nat Genet. 1996;13:417-21.
13. Hewitt JE, Clark LN, Ivens A, Williamson R. Structure and sequence of the human homeobox gene HOX7. Genomics. 1991;11:670-8.
14. Hu G, Vastardis H, Bendall AJ, Wang Z, Logan M, Zhang H, Nelson C, Stein S, Greenfield N, Seidman CE, Seidman JG, Abate-Shen C. Haploinsufficiency of MSX1: a mechanism for selective tooth agenesis. Mol Cell Biol. 1998;18:6044-51.
15. Fraser FC. The genetics of cleft lip and cleft palate. Am J Hum Genet. 1970;22:336-52.



4

Chapter 4

The role of *Msx1* in development

MSX1 and partial anodontia and the Witkop syndrome.

van den Boogaard MJ

Published in: Chapter 75: Inborn errors of development Inborn errors of development : the molecular basis of clinical disorders of morphogenesis / ed. by Charles J. Epstein, Robert P. Erickson, Anthony Wynshaw-Boris. – 2 nd. ed. 2008
ISBN-13: 978-0-19-5360691-0

Msx1, isolated by homology to the *Drosophila* muscle-segment homeobox (*msh*) gene, is highly conserved; it plays an important role in inductive epithelial–mesenchymal interactions, leading to vertebrate organogenesis, and is involved in cell differentiation. During embryonic development, *Msx1* is intensely expressed in the facial primordium and in a variety of embryonic tissues requiring epithelial–mesenchymal interactions for their morphogenesis, such as limb buds and tooth buds.

Msx1 knockout mice exhibit craniofacial defects including cleft palate, abnormalities of the middle ear, defective nail plate, and absent tooth development.

MSX1 mutations in humans are associated with similar phenotypes: partial anodontia, nonsyndromic clefting, and Witkop syndrome (tooth–nail syndrome, OMIM 189500).

The *MSX* genes of vertebrates comprise a small family of homeobox-containing genes. The *msh/msx* gene family is one of the most evolutionarily highly conserved families of homeobox-containing genes and is represented in different species (Table 4.1) (Holland, 1991; reviewed by Davidson, 1995; Bendall and Abate-Shen, 2000; Perry et al., 2005)

Table 4.1 *Msx/Msh* Genes in Different Species **Table 4.1**

Species	Msh-like Gene	References
Human	*Msx1, Msx2*	Ivens et al., 1990; Hewitt et al., 1991; Jabs et al., 1993; Vastardis et al., 1996; Van den Boogaard et al. 2000, 2000; Wilkie et al., 2000; Jumlongras et al., 2001
Mouse	*Msx1, Msx2, Msx3*	Hill et al., 1989; Robert et al., 1989; Monaghan et al., 1991; Bell et al., 1993; Shimeld et al., 1996; Wang et al., 1996
Chicken/quail	*Msx1, Msx2*	Takahashi and Le Douarin, 1990;* Coelho et al., 1991a; Yokouchi et al., 1991; Suzuki et al, 1991; Nohno et al., 1992
Frog *(Xenopus)*	*Msx1, Msx2*	Su et al., 1991
Zebrafish	*MsxA-MsxD*	Ekker et al., 1992; Akimenko et al., 1995
Newt/axolotl	*NvMsx1, AmMsx1, AmMsx2*	Crews et al., 1995; Carlson et al., 1998; Koshiba et al., 1999
Ascidian	*msh*	Holland, 1991
Drosophila	*msh*	Robert et al., 1989
Honeybee	*msh*	Walldorf et al., 1989
Hydra	*msh*	Schummer et al., 1992

*Quail Msx2 was initially thought to be orthologous to murine Msx1/Hox7 and therefore designated as Quox7 in the reference. In later publications, it was renamed Quox8. Msh, muscle segment homeobox.
Source: Davidson (1995), Bendall and Abate-Shen (2000).

Three subclasses can be identified in vertebrates based on a few differences in the homeodomain and neighboring regions of the proteins: *Msx1, Msx2* (formerly *Hox 7.1* and *Hox 8*, respectively; for new nomenclature, see Scott, 1992), and *Msx3.* These genes encode closely related homeodomains.

The observation that *Msx* genes are highly conserved, the specific expression pattern of *Msx1*, its involvement in epithelial–mesenchymal interaction, and the role of MSX1 in the regulation of cellular differentiation by transcriptional repression suggest that Msx1 plays an essential role in vertebrate development (Davidson, 1995; Bendall and Abate-Shen, 2000). This is supported by the observation that mutations in *Msx1* result in congenital anomalies in both mice and humans.

Role of *Msx1* in development

Expression Pattern of *Msx1*

Msx1 is broadly expressed during embryogenesis and organogenesis, and extensive studies of its expression in mouse embryos have been performed (Hill et al., 1989; Robert et al., 1989; MacKenzie et al., 1991a,b; reviewed by Davidson and Hill, 1991; Davidson, 1995; Sadler and Potts, 1997; Houzelstein et al., 1997, 1999, 2000; Bendall and Abate-Shen, 2000; Ramos and Robert, 2005). During embryonic development, *Msx1* is expressed in a variety of tissues in a dynamic manner. The earliest expression in the mouse embryo is detectable in the primitive streak, followed by expression in the neural crest and in cells derived from the neural crest before, during, and after their migration. *Msx1* is also expressed in specific cerebral structures like the ependyma of prosomere 1 and the subcommisural organ during brain development.

Striking expression is seen in craniofacial structures, like the nasal and maxillary processes and mandibular arch, and later in the mouth structures that develop from the arch (Figure 4.1 shows 11.5 days postconception [dpc]). *Msx1* is expressed in the dental papilla and surrounding mesenchymal tissue (Figure 4.2 shows 13.5 dpc).

Figure 4.1

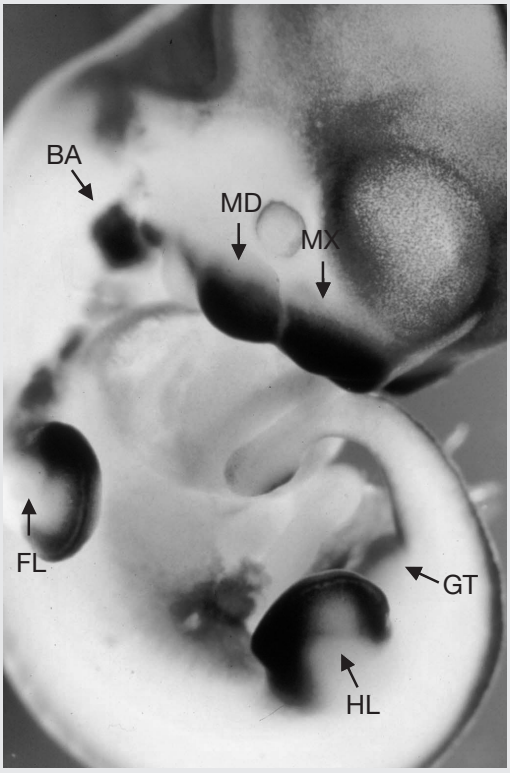


Figure 4.1 Whole-mount heterozygous *Msx1*^{lacZ} mouse embryo stained for b-galactosidase. Stage 11.5 days postcoitum. BA, branchial arch; MD, mandibular process; MX, maxillary process; FL, forelimb bud; HL, hindlimb bud; GT, genital tubercle. (With thanks to D. Houzelstein and B. Robert.)

The developing limb bud is another major site of *Msx1* expression (Figure 4.1). During development, the transcripts become restricted to the distal portion of the developing limb bud, which is essential for limb morphogenesis (Hill et al., 1989; Robert et al., 1989; MacKenzie et al., 1991b; reviewed in Cohn and Tickle, 1996; Houzelstein et al., 1997; Johnson and Tabin, 1997; Bendall and Abate-Shen, 2000).

Expression of *Msx1* has also been reported in the lateral dermomyotome of brachial and thoracic somites, in limb muscle precursor cells migrating to the forelimb, and in a subset

of dermal progenitor cells originating from the somites (Houzelstein et al., 1999, 2000). In addition, *Msx1* is expressed in specific organs such as the heart (Robert et al., 1989; Chan-Thomas et al., 1993) and eye (Monaghan et al., 1991).

Figure 4.2

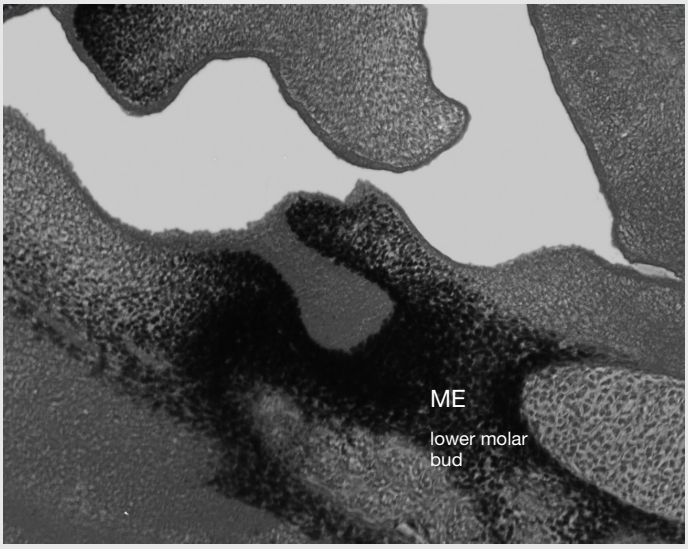


Figure 4.2 *Msx1*^{lacZ} heterozygous mouse embryo stained for b-galactosidase. Stage 13.5 days postcoitum. Magnification of the lower tooth bud. ME, mesectoderm. (With thanks to D. Houzelstein, A. Bach, and B. Robert.)

Postnatal expression of *Msx1* was found in the epidermis (Noveen et al., 1995; Stelnicki et al., 1997), uterus (Pavlova et al., 1994), mammary gland (Friedmann and Daniel, 1996; Phippard et al., 1996), and sutural and bone tissue (Kim et al., 1998; Orestes-Cardoso et al., 2001).

The expression pattern of *Msx1* suggests that it plays an important role in growth, morphogenesis, and organ development, characterized by coordinated growth and cell differentiation in the epithelium and mesenchyme cells (Davidson, 1995; Thesleff et al., 1995). In these processes, there must be a finely regulated balance between proliferation and differentiation. Programmed cell death also contributes to this fine balance (Bendall and Abate-Shen, 2000).

Role of *Msx1* in Apoptosis

The observation of overlapping expression of *Msx1* and *Msx2* (Figure 4.3) preceding programmed cell death, and occurring during embryogenesis, as well as in cell studies suggests that *Msx1* may also participate in apoptosis (Marazzi et al., 1997). For example, both *Msx1* and *Msx2* are both expressed in the neural crest and at the interdigital necrotic zones in the distal limbs (reviewed by Davidson, 1995; Bendall and Abate-Shen, 2000). Several in vivo and in vitro studies confirm that *Msx1* and *Msx2* control and promote cell death during neural crest cell- and neuronal precursor formation in chick and mouse (Takahashi et al., 1998, Tríbulo et al., 2004; Liu et al., 2004; Ramos and Robert, 2005). It was shown that *Msx1* controls the transcription of *Bcl2* and several caspase genes required for programmed cell death (Tríbulo et al., 2004).

Figure 4.3

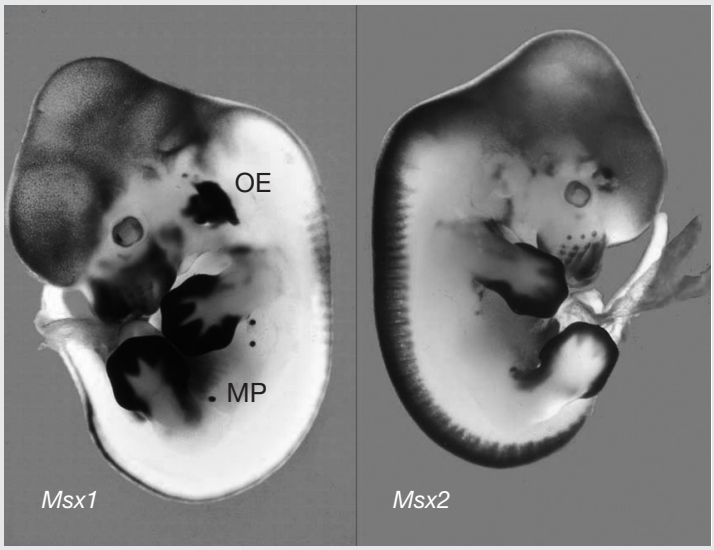


Figure 4.3 Whole-mount heterozygous mouse embryos for *Msx1*^{lacZ} and *Msx2*^{lacZ} stained for b-galactosidase. Stage 12.5 days postcoitum. OE, outer ear; MP, mammary primordium. (With thanks to M.A. Nicola, A. Bach, and B. Robert.)

Msx1 in Epithelial–Mesenchyme Signaling and Pattern Formation

In the mandibular arch and limb processes, *Msx1* transcripts accumulate in zones of ectodermal–mesodermal cell contact; this leads to the hypothesis that *Msx1* is involved

with epithelial–mesenchymal interaction (Robert et al., 1989; Davidson and Hill, 1991). Grafting and tissue recombination studies show that this epithelium–mesenchyme interaction is important in the outgrowth of the limb (Davidson et al., 1991; Robert et al., 1991), outgrowth of facial processes, and correct skeletogenesis in the mandibular arch (Wedden, 1987; Wedden et al., 1988; Richman and Tickle, 1989; Brown et al., 1993; Mina et al., 1995; Francis-West et al., 1998; Bendall and Abate-Shen, 2000; Mina 2001a,b; Alappat et al., 2003; Cox 2004)). Observations in mutant animals also support the hypothesis that *Msx1* is involved in this interaction (Davidson, 1995). Chick mutants showed that signals emanating from the overlying ectoderm control mesenchymal expression of *Msx1* at the distal tip of the limb bud. In the limbless chick mutant, which lacks an apical ectodermal ridge, mesenchymal expression of *Msx1* is reduced but can be restored to normal levels by grafting an apical ridge from a normal embryo (Coelho et al., 1991b). Furthermore, experimental removal of the apical ectodermal ridge in developing chick limb buds showed that the apical ectodermal ridge is a source of instructive signals for *Msx1* expression (Robert et al., 1991).

Tooth and Palate Development and *Msx1*

Tooth development is also regulated by reciprocal interaction between epithelium and mesenchyme (Mina and Kollar, 1987; Lumsden, 1988; Jowett et al., 1993; reviewed in Thesleff et al., 1995; Maas and Bei, 1997; Stock et al., 1997; Thesleff and Sharpe, 1997; Peters and Balling, 1999). Grafting and tissue recombination studies showed that the dental epithelium contains instructive signals for tooth formation and that *Msx1* is expressed in the mesenchyme in response to signals from the overlying epithelium (Mina and Kollar, 1987; Lumdsen, 1988; Jowett et al., 1993; Wang et al., 1998).

For example, expression of the bone morphogenetic protein 4 (*Bmp4*) and fibroblast growth factor 8 (*Fgf8*) in the epithelium induces mesenchymal expression of *Msx1*. MSX1 subsequently induces *Bmp4* expression in the dental mesenchyme and is also involved in the maintenance of BMP4 (Figure 4.4).

Mesenchymal *Bmp4* signaling is involved in the induction of the enamel knot: the transition from the bud to the cap stage. This is an important step in tooth development. Furthermore, *Msx1* is required for expression of *Fgf3* in the dental mesenchyme. FGFs control mesenchymal cell proliferation, which is also an important process in tooth development. After the cap stage, tooth development becomes independent of *Msx1* (Bei et al., 2000).

Of course, many other genes are also involved in tooth development. *Msx1* interacts with several of these genes (Figure 4.4) (Jowett et al., 1993; Vainio et al., 1993; Chen et al.,

1996; reviewed in Thesleff and Sharpe, 1997; Bei and Maas, 1998; Tucker et al, 1998; reviewed in Peters and Balling, 1999; Zhang et al., 1999, 2000; Bei et al., 2000; Bendall and Abate-Shen, 2000; Zhao et al., 2000; Yamashiro et al 2003; Zhang et al., 2003a; Ogawa et al., 2006).

Epithelial-mesenchymal interaction also regulates palatogenesis. *Msx1* appears to control a network of *Bmp* and *Shh* signaling, which induce cell proliferation in the palatal mesenchyme leading to palatal growth (Zhang et al., 2002; Liu et al., 2005, Levi et al., 2006).

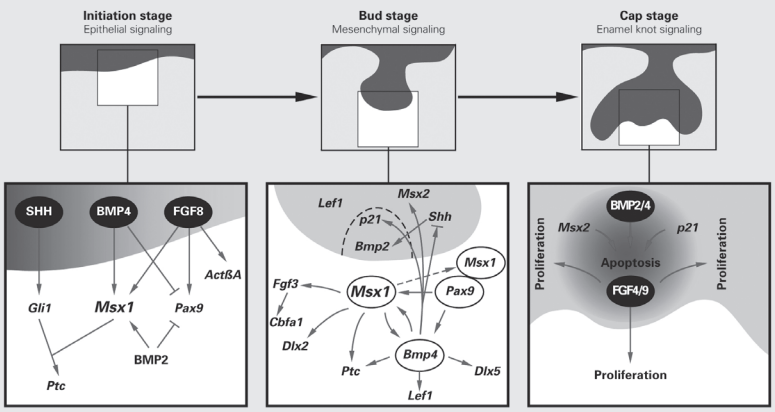


Figure 4.4

Figure 4.4 Schematic representation of reciprocal signaling between the epithelium and mesenchyme during the early phases of tooth development; initiation stage, bud stage, and cap stage. BMP4, bone morphogenetic protein 4; FGF8, fibroblast growth factor 8; SHH, sonic Hedgehog. (Adapted from Peters and Balling, 1999, and Cohen, 2000, with additional data from Zhang et al., 1999, 2000, Zhao et al., 2000; Yamashiro et al 2003; Zhang et al., 2003a; Ogawa et al., 2006).

Msx1 Is Involved in Cellular Differentiation and Proliferation

While *Msx1* is correlated with inductive–epithelial interaction, where coordinated growth and cell differentiation in the epithelium and mesenchyme cell occur, it has been suggested that *Msx1* could also be involved in cell proliferation and cell differentiation (Davidson, 1995). Indeed, the *Msx1* gene is expressed in mesodermal progenitor cells migrating from the primitive streak, where inhibition of cellular differentiation is important (Bendall et al., 1999; Houzelstein et al., 1999).

At a later stage, *Msx1* is associated with specific proliferative zones. For example, in the developing mouse limb bud, there is striking expression of *Msx1* in the proliferative and undifferentiated cells of the progress zone. During limb development, *Msx1* is downregulated in cells that leave the progress zone and differentiate (Hill et al., 1989; Robert et al., 1989; reviewed in Bendall and Abate-Shen, 2000; Hu et al., 2001). Thus, expression demarcates the boundary between undifferentiated cells expressing *Msx1* and differentiated cells not expressing *Msx1* (Odelberg et al., 2000).

Recent studies also indicate a role for *Msx1* in neural development by inhibition of neuronal differentiation and proliferation. *Msx1* overexpression in chick embryos resulted in a smaller neural tube with a specific reduction in neuronal differentiation (Liu et al., 2004; Ramos and Robert, 2005).

Msx1 can also be reinduced during regeneration and is then involved in redifferentiation (Davidson, 1995; Bendall and Abate-Shen, 2000; Han et al., 2005). *Msx1* expression was observed during regeneration in a diverse number of systems, including the fin and heart of the zebrafish (Akimenko et al., 1995; Nechiporuk and Keating, 2002; Raya et al., 2003), fish fin (Murciano et al., 2002), the urodele limb (Koshiba et al., 1998; Odelberg et al., 2000), the anuran limb bud and tail (Yokoyama et al. 2000, Beck et al., 2003) and the mouse digit tip (Reginelli et al., 1995; Han et al., 2003b). The hypothesis that *Msx1* plays a crucial role in cell differentiation can be supported by cell culture data (Hu et al., 2001). Forced expression of *Msx1* in myogenic precursors blocked myogenic terminal differentiation, repressing the expression of lineage-specific genes, like *MyoD*. This resulted in a highly proliferative transformed phenotype (Song et al., 1992; Woloshin et al., 1995). A similar ability to inhibit differentiation was observed in adipocytes, chondrocytes, and osteoblasts (Hu et al., 2001).

There is also evidence that terminally differentiated myotubes or myofibres in both urodeles and mammals can be induced to dedifferentiate to proliferating mononucleated cells by *Msx1*. These cells were capable of redifferentiating into different cell types, expressing characteristic markers of chondrogenesis, adipogenesis, myogenesis, and osteogenesis (Odelberg et al., 2000; Kumar et al., 2004). In addition, ectopic expression of *Msx1* during chicken embryogenesis showed inhibition of the development of limb musculature and repression of *MyoD* in vivo (Bendall et al., 1999).

Msx1 and Proliferative Capacity in Adulthood

Msx1 expression also appears to correlate with the maintenance of proliferative capacity of tissues that are capable of renewal in adulthood (Bendall and Abate-Shen, 2000). In adulthood, *Msx1* is expressed in stem cells of uterine epithelium (Pavlova et al., 1994),

mammary epithelium (Friedmann and Daniel, 1996; Phippard et al., 1996), and basal epithelium of the dermis (Noveen et al., 1995). *Msx1* is also expressed in bone tissue. In bone, progenitor as well as differentiating and differentiated cells of osteoblastic, chondroblastic, osteoclastic, and chondroclastic lineages could express *Msx1* (Kim et al., 1998; Orestes-Cardosa et al., 2001). *Msx1* downregulated *Cbfa1* (core binding factor α1), a master gene of skeletal cell differentiation (Blin-Wakkach et al., 2001; Orestes-Cardosea et al., 2002; Zhang et al., 2003a).

Orestes-Cardosa et al. (2001) proposed that *Msx1* might determine local pools of bone cells in the osteoprogenitor compartment. This could be a result of inhibition of terminal differentiation and enhancement of cell proliferation, which is important for bone growth and homeostasis (Kim et al., 1998). A recent study revealed that *Msx1* is upregulated during mammalian fracture repair (Gersch et al., 2005).

In summary, the expression patterns of *Msx* genes are consistent with a role as general inhibitors of cell differentiation during embryogenesis and maintenance of proliferative capacity of tissues in adulthood, which is important in morphogenesis and growth (Davidson, 1995; Bendall and Abate-Shen, 2000; Hu et al., 2001).

Msx1 Is Involved in the Cell Cycle

Inhibition of cell differentiation due to *Msx1* is correlated with upregulation of *cyclin D1* and cyclin dependent kinase 4 (CDK4*)* activity (Hu et al., 2001). Cyclin D1 inhibits differentiation of multiple cell lineages, by its ability to block the cell from exiting from the cell cycle and undergoing terminal differentiation. Hu et al. (2001) proposed that loss of *Msx1* function would cause premature exit of a cell from the cell cycle and result in differentiation and decreased proliferation, thus impairing growth and morphogenesis. A more recent study by Han et al. (2003a) supported this model by demonstrating that an *Msx1* null mutation results in significant elevated expression of the CDK inhibitor *p19*^{IK4d}. p19^{IK4d} prevents formation of the cyclinD/CDK complex and phosphorylation of Rb proteins, which results in blocking the cell cycle. In the absence of MSX1, cranial neural crest-derived mesenchyme consequently fails to proliferate and misdifferentiates (Han et al., 2003a).

While upregulation of *cyclin D1* is often found in breast carcinoma and since *Msx1* has the potential to upregulate *cyclin D1*, Hu et al. (2001) suggested that *Msx1* may play a role in breast cancer. Park et al. (2001) observed overexpression of *Msx1* in human ovarian cancer cell lines, which suppressed cell proliferation; they considered that this could be due to *Msx1* repressing cell cycle progression. Further study showed that *Msx1* inhibits tumor growth by inducing apoptosis of cancer cells by interaction with the p53 tumor suppressor (Park et al., 2005). These reports support the notion that *Msx1* is involved in tumorigenesis and it deserves further study.

Msx1 Protein Is a Potent Transcriptional Repressor

As already discussed, an important function of MSX1 is to inhibit cellular differentiation. Probable mechanisms are downregulation of master genes (such as *Cbfa1*) and upregulation of genes (such as *cyclin D1*) involved in cell differentiation (Blin-Wakkach et al., 2001; Hu et al., 2001).

The biological function of the homeobox gene *Msx1* was at first described as transcriptional regulation, by DNA binding to specific DNA sequences (Catron et al., 1993). The DNA-binding specificity of MSX1 results from the cumulative action of residues in the N-terminal arm and helices I, II, and III of the homeodomain (Isaac et al., 1995; Zhang et al., 1997; Bendall et al., 1998, 1999; Bendall and Abate-Shen, 2000).

Transcription studies revealed that *Msx* proteins are mainly potent transcriptional repressors (Catron et al., 1995, 1996; Zhang et al., 1996, 1997; reviewed in Bendall and Abate-Shen, 2000). Although the MSX1 homeodomain binds with high affinity to a specific DNA site (containing the TAAT sequence), the homeodomain DNA-binding activity is not required for this transcriptional repression by MSX1 (Catron et al., 1996). *Msx1* appears to repress transcription through protein–protein interactions, mediated by the homeodomain (Zhang et al., 1996). Several models of repression have been discussed by Zhang et al. (1996, 1997) and Bendall and Abate-Shen (2000).

In their review, Bendall and Abate-Shen (2000) proposed that the repression could be a result of direct interaction with the preinitiation complex, blocking the basal transcription machinery by preventing interaction with transcriptional activators. It was demonstrated that *Msx1* proteins could interact with the TATA-binding protein (TBP). TBP is a core component of the basal transcription machinery, which mediates activation (Zhang et al., 1996, 1997; reviewed in Lee and Young, 1998; Bendall and Abate-Shen, 2000). Also, the presence of MSX1 in a multiprotein transcriptional complex containing a basal transcription factor (TBP), a sequence–specific activator (Sp1), and a coactivator (cAMP response element–binding protein–binding protein/p300) was demonstrated by Shetty et al. (1999).

However, the repression may also be due to interaction of the *Msx1* protein with the DNA-bound activator, thereby blocking its function of activating the transcription (Bendall and Abate-Shen, 2000).

Another suggested mode of repression is the interaction of MSX1 with other homeoproteins, preventing them from DNA binding and thereby inhibiting transcriptional activation (Zhang et al., 1997; Bendall and Abate-Shen, 2000). Heterodimer formation between the homeoproteins MSX and DLX (a transcriptional activator) results in functional antagonism (Zhang et al., 1997). Furthermore, heterodimer formation between MSX1

and genes coding for members of the LIM and PAX families prevents DNA binding of MSX1 and the other protein involved (Zhang et al., 1997; Bendall et al., 1998, 1999; Bendall and Abate-Shen, 2000).
Interestingly, the *Lhx2* LIM-homeobox gene (functionally interchangeable with the ap gene in *Drosophila*) and *Msx1* expression patterns in the mouse are complementary in most tissues. However, in the developing limb, they are coexpressed (Lu et al., 2000). In *Drosophila*, this spatial relationship of expression patterns for *ap* and *msh* was also recognized. The expression patterns of both genes in the mouse and *Drosophila* suggest that the regulation of these genes has been conserved during evolution (Lu et al., 2000). In *Drosophila*, the *msh* gene acts downstream of *ap* and is involved in dorsal identity specification in wing development (Milan et al., 2001).
In vivo, complex formation between MSX1 and PAX3 may prevent premature activation of myogenic genes (*MyoD*) in migratory limb muscle precursor cells during their migration (Bendall et al., 1999). Recent studies show that MSX1 forms a complex with histone isoform H1b, which binds to the enhancer of the *MyoD* gene. This blocks the expression of *MyoD*, thereby preventing the differentiation of muscle progenitor cells (Cirillo and Zaret 2004; Lee et al., 2004).

There is also a relationship between *Msx1* and *Pax9*, at both transcriptional and protein levels, in the regulation of *Bmp4* expression, which is essential in tooth morphogenesis (Ogawa et al., 2006).
Msx1 also plays a role in neural development by repressing regulatory genes (Liu et al., 2004; Ramos and Robert 2005).

Regulation of *Msx1* Expression

A finely tuned regulation of *Msx1* is important for balanced cell growth and differentiation, and *Msx1* appears to be involved in the regulation of its own transcription. The *Msx1* promotor may itself be subject to *Msx1*-mediated transcriptional repression. Thus, *Msx1* has an autoinhibitory activity (Shetty et al., 1999).
Another mechanism is the presence of antisense (AS) RNA. Involvement of endogenous AS RNAs in regulating gene expression has been documented for various genes and, in most cases, regulation occurs at the translational level. The AS transcript hybridizes to the sense transcript and blocks access of the translation machinery to the sense transcript. This leads to reduced levels of protein synthesis (reviewed in Inouye, 1988; Kumar and Carmichael, 1998). The presence of endogenous *Msx1* AS RNA (Msx1-AS RNA) has been demonstrated in mice, rats, and humans (Blin-Wakkach et al., 2001;

Berdal et al., 2002). The *Msx1*-AS cDNA is complementary to the region extending from the 3' end of exon 2 to the middle of intron 1 of the genomic *Msx1* DNA sequence. In vivo data showed that the balance between the levels of *Msx1* RNA and *Msx1*-AS RNA is related to expression of the *Msx1* protein (Blin-Wakkach et al., 2001).

New mechanisms for modulating the molecular function of *Msx1* were proposed by Gupta and Bei (2006) and Lee et al. (2006). They reported that *Msx1* interacts with *PIAS1*, which results in modification of MSX1 by the small ubiquitin-related modifier SUMO-1. Lee et al. (2006) showed, however, that the key role of PIAS1 is in regulating the DNA binding specifity on the *Msx1* protein by regulating the subnuclear localization and proximity of *Msx1* to target genes. Sumoylation probably plays a role in the fine-tuning of this mechanism.

Clinical Features Associated with *MSX1* Mutations in Humans

Mutations in *MSX1* have been reported in seven families with an autosomal dominant form of hypodontia [OMIM 106600] (Vastardis et al., 1996; van den Boogaard et al., 2000; Jumlongras et al., 2001; Lidral and Reising, 2002; De Muynck et al., 2004; Kim et al., 2006; Mostowska et al., 2006) (see table 4.2).

Table 4.2

Table 4.2 Published Autosomal Dominant *MSX1* Coding Region Mutations in Families with Tooth Agenesis and/or Orofacial Clefting

				Phenotype		
Reference	Mutation	Previously known as	Type of Mutation	Tooth agenesis	Oral Clefting	Nail Defect
Vastardis et al., 1996	p.Arg202Pro	R196P	Missense	1	2	2
Van den Boogaard et al., 2000	p.S111*	S105X	Nonsense	1	1	2
Jumlongras et al., 2001	p.Ser208*	S202X	Nonsense	1	2	1
Lidral and Reising, 2002	p.Met67Lys	M61K	Missense	1	2	2
De Muynck et al., 2004	p.Gln193*	Q187X	Nonsense	1	2	2
Suzuki et al., 2004	p.Pro153Gln	P147Q	Missense	2	1	2
Vieira et al., 2005	p.Pro153Gln	P147Q	Missense	2	1	2
Kim et al., 2006	p.Gly28Argfs*147	G22RfsX168	Frameshift	1	2	2
Mostowska et al., 2006	p.Ala200Val	A194V	Missense	1	2	2

1, present ; 2, absent

The first family described had an autosomal-dominant form of hypodontia [OMIM 106600] caused by an p.Arg202Pro missense mutation in *MSX1* (Vastardis et al., 1996). Affected individuals most frequently showed agenesis of the maxillary and mandibular second premolars; some affected individuals missed the maxillary first premolars and mandibular first molars. Skull X-rays showed loss of proper tooth inclination in relation to the jaws. The maxilla was also slightly shorter than the mandibula, although both were within normal limits. Affected individuals showed no craniofacial abnormalities, nor were nail or limb defects mentioned.

Analysis of the data from all seven families with hypodontia and an *MSX1* mutation showed that, as in the first family described, the maxillary and mandibular premolars (91% and 97% respectively) and the first premolar (75%) were most frequently absent (Kim et al., 2006).

In addition, mutations of *MSX1* have been associated with a combination of tooth agenesis with orofacial clefting or nail abnormalities.

In a Dutch family, an *MSX1* nonsense mutation (p.Ser111*) was associated with familial tooth agenesis and various combinations of cleft lip and cleft lip/palate (nonsyndromic cleft lip with or without cleft palate [OMIM 119530]/isolate cleft palate [OMIM 119540]) (van den Boogaard et al., 2000). There were 12 affected members in this family (Figure 4.5).

Four males had a cleft. The 5-year-old proband (IV-1) and a maternal uncle (III-4) had a cleft palate. II-3 had a cleft alveolar ridge, and III-9 had a cleft lip and palate. Eleven family members lacked some permanent teeth (Table 4.3).

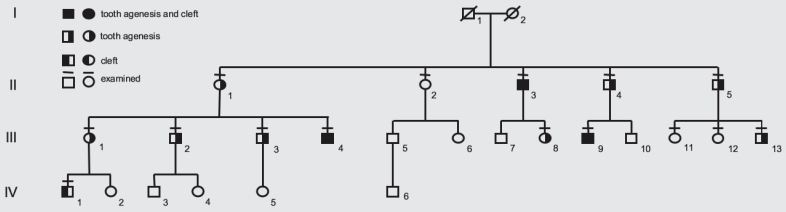


Figure 4.5

Figure 4.5 Pedigree and symptoms of the Dutch family with the Ser111* *MSX1* mutation.

Table 4.3

Table 4.3 Pattern of Tooth Agenesis in a Dutch Family with p.Ser111* *MSX1* Mutation Associated with Clefting and Tooth Agenesis

Pedigree	Dental	Right								Left							
ID	Arch	8	7	6	5	4	3	2	1	1	2	3	4	5	6	7	8
II-1	Maxillary														*		
	Mandibular			*					*	*							
II-4	Maxillary	*			*									*			*
	Mandibular	*			*									*			*
II-5	Maxillary	*			*	*								*			*
	Mandibular	*			*									*		*	*
III-1	Maxillary	*			*	*							*	*			*
	Mandibular	*			*									*			*
III-4	Maxillary				*									*			
	Mandibular				*									*			
III-8	Maxillary				*	*		*			*		*	*			
	Mandibular																
III-9	Maxillary				*	*		*					*	*			
	Mandibular	*			*									*			*
III-13	Maxillary	*	*		*			*			*			*		*	*
	Mandibular	*	*		*								*	*	*	*	*

*Missing tooth; 1, central incisor; 2, lateral incisor; 3, canine; 4 and 5, first and second premolars, respectively; 6, 7, and 8, first, second, and third molars, respectively.

The pattern of tooth agenesis was almost identical to that of the first family described with an *MSX1* mutation. Most affected individuals were missing both mandibular and maxillary second premolars. The third molar was also frequently absent. In most cases, the pattern of dental agenesis was bilaterally symmetrical. As far as we know, the affected family members showed no nail defects.

Another *MSX1* mutation (p.Ser208*) was identified in a three-generation family with familial tooth agenesis in combination with dysplastic toenails and/or fingernails, diagnosed as tooth-and-nail syndrome, also known as Witkop syndrome or nail dysgenesis and hypodontia (Jumlongras et al., 2001).

No orofacial cleft or any other craniofacial abnormalities were present in affected members of this family. However, affected individuals had a large number of congenitally missing permanent teeth (11–28), which also showed a specific pattern. The premolars, first molars, and third molars were predominantly absent. In a few cases, other teeth, like incisors and canines, were also missing. The permanent teeth that were present appeared smaller in the mesiodistal dimension and had shorter root lengths than normal. The primary dentition was normal, except in one affected individual in whom the mandibular right primary central and lateral incisor was fused. Affected members had a prominent maxillar frenulum, and frenectomy was required to accommodate dental prostheses.

In most cases, nail dysplasia was present. The toenails were more severely affected, especially the fifth toenail. The skin, sweat glands, and hair were normal.

MSX1 substitutions have also been reported in individuals with isolated nonsyndromic clefting (Lidral et al., 1998; Jezewski et al., 2003; Suzuki et al., 2004; Vieira et al., 2005). A p.Pro153Gln was detected in three Vietnamese families with nonsyndromic cleft lip and/or palate and two indivuals with clefting from the Philippines. This variant resulted in variable expression and decreased penetrance.

Partial Anodontia

The tooth phenotype in families with an *MSX1* mutation is different from common hypodontia. The reported frequencies of hypodontia in the general population lie within the range 1.6%–10.1%. This figure excludes the absence of third molars, which occurs in 20% of the population (Graber, 1978; Schalk-van der Weide, 1992; Cohen, 2000). The most commonly missing permanent teeth are premolars and maxillary lateral incisors (Symons et al., 1993), for which *MSX1* and *MSX2* have been excluded as candidate genes (Nieminen et al., 1995).

Interestingly, the reported *MSX1* mutations mainly seem to affect the maxillary and

manbibular second premolars and the maxillary first premolar. The first and third molars were also frequently absent (Line 2003; Kim et al., 2006). Several authors have discussed the possible cause of this selective tooth agenesis (Vastardis et al., 1996; Thesleff, 1996; Peters and Balling, 1999; Jumlongras et al., 2001; Vastardis 2000).

The basic genetic mechanisms of tooth development are conserved between different tooth classes. The teeth within a class evolve as a unit. Several models have been proposed to explain the differences in shape between tooth classes. One model presumes a prespecification of neural crest cells, which determine the identity of each individual tooth. Another model suggests that different tooth types develop from initially identical tissue and that the differences in development are due to influences from the molecular environment. There can be positional differences between the gradients and presence of other homeoproteins, resulting in different tooth types (Stock et al., 1997; Peters and Balling, 1999). Indeed, ectopic expression of *Barx1* in the distal presumptive incisor mesenchyme, due to inhibition of *Bmp4* signaling, results in transformation of tooth identity from incisor to molar (Tucker et al., 1998). These findings support the idea that homeobox genes are involved in the determination of different tooth types.
It was suggested that the timing of *Msx1* expression and the presence or absence of possible redundancy from other genes could be critical for the development of specific teeth (Thesleff, 1996; Jumlongras et al., 2001) and could explain the specific pattern of tooth agenesis associated with *MSX1* mutation.
Furthermore, the missing teeth are the last to develop from each tooth class and might therefore be more susceptible to defects during development than the other elements (Thesleff, 1996; Line 2003).

Orofacial Clefting

In one family with an *MSX1* nonsense mutation four affected individuals had an orofacial cleft (van den Boogaard et al., 2000). Additionally, three Vietnamese families with clefting and two individuals with a cleft from the Philippines showed an *MSX1* substitution (Suzuki et al., 2004; Vieira et al., 2005).
In general, the prevalence of orofacial cleft at birth varies from 1 in 500 to 1 in 2500, depending on ethnic background, geographic origin and socioeconomic status. The etiology of orofacial clefting is complex, and several genetic and environmental factors are involved (Schutte and Murray, 1999; Spritz, 2001; Murray, 2002).
Genetic epidemiological studies suggest that several interacting loci, including a major gene, are involved in the etiology of orofacial clefting and may account for approximately

half of the familial occurrences (FitzPatrick and Farral, 1993; Christensen and Mitchell, 1996). Several candidate genes (e.g. *MTHFR*, *TGFA*, *BCL3, DLX2*, *MSX1*, and *TGFB3*) have been screened for linkage disequilibrium with clefting. Various linkage and association studies have shown that the risk of orofacial clefting may be influenced by variation at the locus for *MSX1* (Lidral et al., 1998; Beaty et al., 2001; Blanco et al., 2001; Beaty et al., 2002; Fallin et al., 2003; Jugessur et al., 2003; Slayton et al., 2003; Vieira et al., 2003; Marazita et al., 2004; Moreno et al., 2004; Schultz et al., 2004; Suazo et al., 2004; Modesto et al., 2006). Detection of an *MSX1* mutation in individuals with orofacial clefting confirmed *MSX1* as a candidate gene for orofacial clefting (van den Boogaard et al., 2000). The result of direct sequencing of *MSX1* in a large population with clefting suggests that point mutations in this gene underlie approximately 2% of cases with nonsyndromic clefting (Jezewski et al., 2003; Suzuki et al., 2004).

Previous studies suggested an etiological distinction between isolated cleft palate and cleft lip, with or without cleft palate (Fraser, 1955, 1970). However, cases of cleft palate only, and of cleft lip and cleft palate, were seen in the second *MSX1* family. This mixed clefting phenotype is also noted in families affected with the syndromic forms of orofacial clefting: Van der Woude's syndrome and ectrodactyly–ectodermal dysplasia–clefting. These syndromes are caused by mutations in the *IRF6* and *TP63* genes, respectively (Celli et al., 1999; McGrath et al., 2001; Kondo et al., 2002). Kondo et al. (2002) suggested that both these genes and MSX1 are involved in common genetic pathways. Interestingly, a recent study revealed that *P63* and *Msx1* are both part of *Bmp4* signaling pathways and it is known that *Bmp* signaling plays a role in orofacial clefting (Liu et al., 2005).

In humans, tooth agenesis is noted in about 35% of individuals with isolated cleft palate, despite the fact that the cleft does not directly involve the tooth-bearing area (Ranta, 1986). This suggests that odontogenesis and palate formation are developmentally related events and that a single gene could be involved in both processes. Since *MSX1* is associated with both tooth agenesis and orofacial clefting, it might be such a single gene.

Witkop Syndrome (Tooth-and-Nail Syndrome)

In one family, the *MSX1* mutation is associated with features of Witkop syndrome (Jumlongras et al., 2001). This family was first described in 1997 by Stimson et al.

Witkop (1965) described autosomal-dominant hypoplasia of the nails with hypodontia. This combination of symptoms was common in the Dutch Mennonites of Canada (Chitty et al., 1996). The combination of hypodontia with dysplastic nails was first reported by

Weech (1929), though more fully by Witkop (1965) and Witkop et al. (1975) (see Murdoch-Kinch et al., 1993). The incidence has been estimated to be approximately 1 to 2:10,000 (Witkop, 1990). Since the original communication, several other families and cases with Witkop syndrome have been reported (Redpath and Winter, 1969; Giansanti et al., 1974; Hudson and Witkop, 1975; Murdoch-Kinch et al., 1993; reviewed by Chitty et al., 1996; Garzon and Paller, 1996; Stimson et al., 1997; Zabawski and Cohen, 1999; Hodges and Harley, 1999; Jumlongras et al., 2001).

The main features of Witkop syndrome are a variable number and variable types of congenitally missing permanent teeth. The teeth may be widely spaced. Anodontia is rare in this disorder but has been reported (Giansanti et al., 1974). The deciduous teeth may be normal, but there are some reports of congenitally missing deciduous teeth (Chitty et al., 1996). Tooth shape may also be conical.

The nails are generally thin, slow-growing, brittle, and spoon-shaped. In most cases, the toenails are more severely affected than the fingernails.

The phenotypic expression, including tooth and nail defects, seems, however, to be highly variable. In some instances, the only features are marked longitudinal ridges and pitting (Giansanti et al., 1974). The nail defects improve with age, so these may not be detectable in adulthood. Older children and adults frequently have normal fingernails but small, spoon-shaped toenails.

The hair has a normal distribution, but it may be fine and slow-growing. Sparse eyebrows have been described in a few affected individuals (Chitty et al., 1996).

Affected individuals have no typical facial phenotype. However, there may be a small jaw, eversion of the lower lip as a result of the tooth agenesis, and maxillary hypoplasia.

One family with only one affected individual with a cleft palate has been reported (Chitty et al., 1996). We can speculate whether this is part of the phenotypic expression of a probable *MSX1* mutation in this family. In this case, the phenotypes between the *MSX1* families show more overlap and are less distinguishable (see Table 4.2). Also, because the deciduous teeth may be normal and the nail abnormalities may be very mild in Witkop syndrome and since these symptoms could have been missed in the other *MSX1* families, the overlap in phenotypes might be more obvious than previously suggested (Jumlongras et al., 2001).

Other Symptoms of the Spectrum

Because there are only a few families in which an *MSX1* mutation has been identified and since they were selected on the presence of tooth agenesis, we cannot exclude that *MSX1* is involved in a range of other phenotypes. *MSX1* mutations may result in

abnormalities of other structures or tissues where *MSX1* is expressed and may be associated with less prominent abnormalities in tooth development. For example, during development, *Msx1* is expressed in the central nervous system (CNS) and some of the *Msx1* homozygous mutants display hydrocephaly at birth (Bach et al., 2003; Fernández-Llebrez et al., 2004; Ramos et al., 2004); Ramos and Robert, 2005). So, an *MSX1* mutation may be related to an abnormal morphology of specific brain structures, although this has not been established in humans.

In addition, it is well known that a different pathogenic effect of mutations can result in a different phenotype. Genotype–phenotype studies of the *MSX2* gene provide an example of this: loss-of-function mutation in *MSX2* results in defective cranial osteogenesis and enlarged parietal foraminae, while a gain-of-function results in premature cranial suture differentiation, ossification, and hence craniosynostosis (Ferguson, 2000; Cohen, 2000). To date, no gain-of-function mutations in *MSX1* have been reported.

However, it has been suggested that overexpression of *MSX1* may also play a role in mammary carcinoma and ovarian cancer (Hu et al., 2001; Park et al., 2001). Thus, the recognized phenotype of *MSX1* mutations could be a result of selection bias. To elucidate the possible phenotypic expression of *MSX1* mutations, it is necessary to identify more families with *MSX1* mutations and to study other congenital abnormalities that could be related to *MSX1*.

Molecular genetics of *MSX1*

Structure of *Msx1*

The murine homologue of *Msh*, *Msx1* (formerly *Hox7*), was characterized and mapped to mouse chromosome 5 (Hill et al., 1989; Robert et al., 1989). In 1990, the human locus was mapped to chromosome 4 (4p16.1), a region thought to be involved in Wolf-Hirschhorn syndrome (Ivens et al., 1990). Subsequently, the structure and sequence of the human homeobox gene *MSX1* were reported by Hewitt et al. (1991). The homeodomain of 60 amino acids is highly conserved, consisting of an N-terminal arm and helices I, II, and III. Human and murine Msx1 show 94% identity in the homeodomain at the DNA level. Homology at the nucleotide coding level of Msx1 was 80%, and 100% for the homeodomain. *Msx1* also contains a conserved noncoding sequence, including a strech of 56 bp that is 100% conserved (Hewitt et al., 1991; Padanilam et al., 1992; Perry et al., 2006)).

Sequence analysis of the human *MSX1* gene showed that it has two exons separated by an intron of approximately 1.6 kb. The intron is located 40 bp upstream of the homeobox, a position comparable to that in other homeobox genes (Hewitt et al., 1991). Exon 2 contains the homeobox.

The gene is approximately 4 kb. The human *MSX1* cDNA is 1940 bp long. The gene encodes a protein of 303 amino acids, and the homeodomain extends from residues 170 through 234. *MSX1* has a putative GC-rich promotor region. The 59 upstream region does not contain a TATA box, although an AT-rich region is present with a CCAAT box upstream. There are several GC boxes.

Comparing the *Msx1* cDNA sequence from five species revealed a strictly conserved, 66 bp region at the 39 noncoding end that contains a consensus TATA box, identified as the initiation site of *Msx1*-AS RNA transcription (Blin-Wakkach et al., 2001). *Msx1* codes for a DNA-binding protein, which functions as a transcriptional repressor through its interaction with general transcription factors and other homeoproteins. The homeodomain is essential for these functions and important for protein stability (Hu et al., 1998).

MSX1 Mutations in Humans

Eight heterozygous *MSX1* coding region mutations have been reported in unrelated families (Table 4.2, Figure 4.6) (Vastardis et al., 1996; van den Boogaard et al., 2000; Jumlongras et al., 2001; Lidral and Reising, 2002; De Muynck et al., 2004; Suzuki et al., 2004; Vieira et al., 2005; Kim et al., 2006; Mostowska et al., 2006)).In these families, affected individuals are heterozygous for an autosomal-dominant mutation.

Seven mutations caused selective tooth agenesis [OMIM 106600]. One of these mutation (p.Ser111*) was also associated with orofacial cleft and one mutation (p. Ser208*) with nail dysplasia.

All but one of the mutations that cause tooth agenesis occur within or disrupt the homeobox domain, which is highly conserved. Three mutations (p.Ser111*, p.Gln193*, p.Ser208*) are nonsense mutations and one mutation (p.Gly28Argfs*168) results in a premature stop by a frameshift. The two missense mutations (p.Arg202Pro) and (p. Ala200Val) mutations are located in the homeobox coding region in exon 2 and occur within helix II. This helix, like the N-terminal arm and helix I, is very important for protein stability (Shang et al., 1994; Isaac et al., 1995; Zhang et al., 1996, 1997; Hu et al., 1998). The mutation that did not disrupt the homeodomain was located in exon 1 (p.Met67Lys). Interestingly, this mutation appears to be located within a region that is 100% conserved at the amino acid level across all primates and mice (Perry et al., 2006).

Nearly all of the mutations that have been detected in populations with orofacial clefting

were located in either exon 1 or in noncoding regions (Lidral et al., 1998; Jezewski et al., 2003, Suzuki et al., 2004). Perry et al. (2006) found 100% conservation across the primates included in their study for these mutations too.

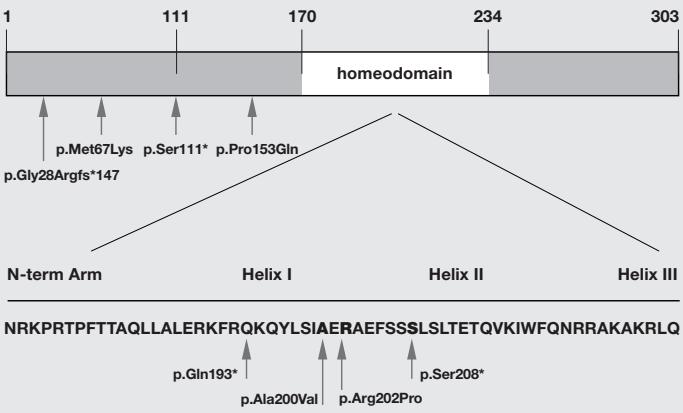


Figure 4.6

Figure 4.6 Simple schematic diagram of *MSX1* showing the published autosomal dominant *MSX1* mutations in families with tooth agenesis and/or orofacial clefting.

4

Phenotype–Genotype Correlation

The *MSX1* mutations show overlapping and nonoverlapping phenotypes. All the mutations but one (p.Pro153Gln) are responsible for autosomal-dominant selective tooth agenesis of especially the second premolars, first molars, and third molars. The *MSX1* mutations p.Ser111* and p.Ser208* are also associated with combinations of tooth agenesis with oral clefting and tooth agenesis with nail defects (Witkop syndrome), respectively. Because of the varying degree of nail abnormalities or even the absence of nail abnormalities in Witkop syndrome, this additional symptom could have been missed or simply not present in the other families described. The *MSX1* mutation p.Pro153Gln is associated with clefting and shows incomplete penetrance.

The presence or absence of nail defects or orofacial clefting might be related to the type of mutation and other possible modifying factors (sense/AS, molecular environment, genetic background, environmental factors). The phenotype of *MSX1* mutation probably represents only one spectrum of symptoms, including selective tooth agenesis, orofacial clefting, and nail abnormalities.

Haploinsufficiency

Haploinsufficiency is the probable cause for the phenotype of six mutations associated with tooth agenesis. The effect of the *MSX1* (p.Arg202Pro) mutation was studied by biochemical and functional analyses (Hu et al., 1998). *MSX1* (p.Arg202Pro) has a perturbed structure and reduced thermostability compared with wild-type *MSX1*, resulting in severe impairment of biochemical activities. There is little or no ability to interact with DNA or other protein factors, and *MSX1* (p.Arg202Pro) does not have the ability to act as a transcription repressor. *MSX1* (p.Arg202Pro) is inactive in vivo. The effect of MSX1 (p.Arg202Pro) was examined in developing chicken limb buds but not in developing teeth. Biochemical studies demonstrated that MSX1 (p.Arg202Pro) has no apparent novel activities. Since MSX1 (p.Arg202Pro) appears to be inactive, haploinsufficiency is the most likely pathogenic mechanism for the tooth agenesis in the family with the p.Arg202Pro mutation (Hu et al., 1998). Because the p.Ala200Val is located only 2 amino acid residues from the p.Arg202Pro, this mutation may lead to a similar effect on the gene product resulting in haploinsufficency (Mostowska et al., 2006). The p.Gly28Argfs*147 would lead to a truncated protein, and only the 21 aminoacids would be the same as in the native protein (Kim et al., 2006). p.Ser111* is a stop mutation proximal to the homeodomain in exon 2. Both mutations probably lead to haploinsufficiency (van den Boogaard et al., 2000).

The p.Gln193* and Ser208* mutations both lead to a truncated protein, missing part of the homeodomain and the C-terminal end. This protein is probably not properly folded, is unstable, or is unable to bind to DNA (Jumlongras et al., 2001). However, we can hypothesize that p.Gln193* and Ser208* are stable and the N-terminal arm of the homeodomain still has some function.

Although *Msx1* has been well studied and haploinsufficiency is most likely to be the pathogenic mechanism in most of the mutations, there remains much to be discovered about its mode of action (Bendall and Abate-Shen, 2000). The p.Met67Lys also leads to tooth agenesis. Interestingly, this missense mutation is located in a region that is 100% conserved and near a putative sumoylation site in *MSX1*. We cannot exclude that the different mutations may have diverging effects on pathways still to be elucidated and on mechanisms that modulate the *MSX1* function.

Sense–AntiSense RNA

Msx1 expression can be regulated by the balance between the levels of *Msx1* RNA and *Msx1*-AS RNA. The ratio of both *Msx1* RNAs appears to be a key factor for cell differentiation and phenotypic expression in mineralized tissues (Blin-Wakkach et al.,

2001; Berdal et al., 2002; Coudert et al., 2005). However, what effect do mutations in *Msx1* have on the balance of the two *Msx1* RNAs and expression of the Msx1 protein? When mutations can interfere in this mode of gene regulation, the sense/AS mechanism could be an important element of the pathogenic mechanism and could explain the gradational effects seen in different mutations. We can speculate if the position of the mutation, in exon 1 versus exon 2, plays a role in its effect on the ratio of both *Msx1* RNAs. A nonsense mutation in the 5' site of the gene might be associated with absent mRNA, which could influence the ratio of *Msx1* RNA and *Msx1*-AS RNA. Further studies are needed to evaluate these possible mechanisms.

Variable Expression

We see great intrafamilial and interfamilial clinical variability in families carrying an *MSX1* mutation. This clinical variability could be explained by other genes acting in the same or in different developmental pathways, possibly as modifiers on the effect of the MSX1 mutation. The biological function of the *MSX1* protein might also be context-dependent and influenced by the molecular environment of the tissue in which it is expressed (Bendall and Abate-Shen, 2000; Gupta and Bei, 2006; Lee et al., 2006). Many cell adhesion molecules, extracellular matrix components, and cell surface matrix receptors have been associated with morphogenesis (Thesleff et al., 1995). Differences in genetic background might also be responsible for the phenotypic differences. The families originate from different countries. *MSX1* may also be involved in sex-dependent susceptibility to orofacial clefting (Blanco et al., 2001).

In addition to potential modifier genes, molecular environment, and genetic background, environmental factors may modulate the clinical expression. Smoking and alcohol consumption during pregnancy appeared to be associated with an increased risk for cleft palate and cleft lip with or without cleft palate, respectively, if the infant had an allelic variant of the *MSX1* site (Romitti et al., 1999). Another study provided evidence for an interaction between infants' *MSX1* genotype and maternal smoking (Beaty et al., 2002).

Diagnosis

The main features of the phenotypes associated with *MSX1* mutation are selective tooth agenesis, probably in combination with nail abnormalities or orofacial clefting. The developmental absence of the maxillary and mandibular second premolars and maxillary first premolar, while the mandibular first premolar is present, appears the pattern of tooth

agenesis that indicates an *MSX1* mutation. The absence of the maxillary first premolar is the most distinguishing feature of an *MSX1* mutation compared to a *PAX9* mutation (Kim et al., 2006). Since the tooth abnormalities may not be present in childhood and the nail defects improve with age, it may be difficult to recognize these symptoms as part of the phenotypic spectrum of the *MSX1* phenotype. Furthermore, nail and tooth abnormalities are common symptoms of several forms of ectodermal dysplasia, a heterogeneous group of disorders characterized by defects in at least two ectodermally derived organs, such as teeth, nails, hair, and sweat glands (Slavkin et al., 1998).
However, the *MSX1* mutation phenotype can be distinguished from several forms of ectodermal dysplasia by the presence of only teeth and/or nail abnormalities, and the features do not meet the criteria of specific ectodermal dysplasias. In addition, *MSX1* mutation seems to affect only specific types of permanent teeth: premolars, first molars, and third molars. When clefting is present, other causes or syndromes have to be excluded since clefting is a feature of more than 400 syndromes. A careful family history must be obtained to confirm an autosomal-dominant pattern for this disorder because of its variable expression.

Management

Abnormal dentition seems to be the most common feature of the *MSX1* mutation. Affected individuals have a variable number and variable types of congenitally absent permanent teeth. Early correction of dental abnormalities is essential in the management of this disorder. Surgical repairs for various clefts of the lip and palate may also be indicated.
Another aspect of patient care is counseling of affected individuals and family members because the inheritance is autosomal-dominant. Since there may be considerable variation in expression, gene carriers may have only minimal signs, but they can be identified by mutation analysis once a pathogenic mutation has been identified in the family.

Knockout mouse

To determine the phenotypic consequences of deficiency of *Msx1*, two homozygous *Msx1*$^{-/-}$ mice were created (Satokata and Maas, 1994; Houzelstein et al., 1997). In the first knockout, a *PMC1neo* gene was introduced into helix III of the homeodomain,

truncating the gene in the homeodomain (Satokata and Maas, 1994). In the second knockout, a reporter *nlacZ* gene, containing a nuclear localization signal (n), was introduced at the same restriction site where the *PMC1neo* gene was introduced in the first knockout. While the mutated gene forms an Msx1–b-galactosidase fusion protein, *Msx1* expression could be studied by b-galactosidase histoenzymology (Houzelstein et al., 1997).

Homozygous mice died a few hours after birth and exhibited marked congenital abnormalities. They failed to form teeth and had craniofacial defects, with absence of the alveolar bones in the mandible and maxilla and lesser abnormalities in the parietal, frontal, and nasal membrane bones; cleft of the secondary palate; and abnormalities of the malleus in the middle ear. They also exhibited defective nail plates (Jumlongras et al., 2001) and brain abnormalities (Ramos and Robert, 2005).

It was striking that most other sites where *Msx1* is strongly expressed appeared to be normal, and several possible explanations have been suggested (Satokata and Maas, 1994; Davidson, 1995; Thesleff et al., 1995; Catron et al., 1996; Houzelstein et al., 1997). First, the phenotype reported may not represent the real null mutation. In the knockouts, the mutation truncates the gene in the homeodomain. Thus, the N-terminal arm of the homeodomain is still present, which might leave residual activity of the protein (Davidson, 1995). However, the mutations may nevertheless be null because of the disturbance of the three-dimensional structure of the mutant protein (Houzelstein et al., 1997).

Second, expression in the unaffected tissues in the knockout mice may be superfluous (Thesleff et al., 1995). *Msx1* may have no function at all in these tissues (Houzelstein et al., 1997). In this respect, expression of AS *Msx1* may play a role.

Third, other cofactors are necessary for the proper functioning of MSX1, and MSX1 is nonfunctional in sites where the cofactor is not expressed or absent (Houzelstein et al., 1997).

Several authors have suggested that the absence of phenotypic alterations in tissues expressing *Msx1* could be explained by functional redundancy of *MSX1* and *MSX2* (Satokata and Maas, 1994; Catron et al., 1996; Houzelstein et al., 1997). Analysis of *Msx1/Msx2* compound mutants revealed that absence in both genes resulted in earlier and stronger phenotypes, particularly in skull bones, cranial ganglia, pharyngeal arch derivatives, middle ear, ectodermal organs, abdominal wall, limbs and heart (Bach et al., 2003; Zhang et al., 2003b; Ishii et al., 2005; Lallemand et al., 2005; Ogi et al., 2005). Although *Msx1* and *Msx2* are expressed in overlapping patterns, there are discrete differences (Fig. 4–3) (Catron et al., 1996; Houzelstein et al., 1997; Sadler and Potts, 1997; M.-A. Nicola, unpublished data). *Msx2* expression in the frontonasal process is much more restricted than expression of *Msx1*. With respect to tooth development,

Msx2 is preferentially expressed in dental ectoderm and *Msx1* is expressed in dental mesenchyme (MacKenzie et al., 1991a,b, 1992; Chen et al., 1996; Maas et al., 1996; Houzelstein et al., 1997; Thesleff and Sharpe, 1997). With respect to early limb development, the expression pattern shows considerable overlap, although the exact level of expression of *Msx1* and *Msx2* differs (Hill et al., 1989; Coelho et al., 1991a,b; Robert et al., 1991; Yokouchi et al., 1991; Nohno et al., 1992; Catron et al., 1996; Houzelstein et al., 1997; Bendall and Abate-Shen, 2000).

In the limbs, expression of *Msx1* and *Msx2* seems sufficiently similar to permit MSX2 to compensate for MSX1, but in the facial region and the teeth, this is not the case. It is proposed that the redundancy may be achieved through different mechanisms, which have similar functional outcomes (Catron et al., 1996; Houzelstein et al., 1997). Analyses show that *MSX1* and *MSX2* have similar DNA-binding and transcriptional properties but that these functions are modulated differently by their nonconserved N-terminal regions. For some pathways, other proteins with similar functions may compensate for *MSX1*. The regulation of expression of proteins where *MSX1* is involved might be subject to additional mechanisms, which compensate for the absence of *MSX1*.

Developmental pathogenesis of the condition

To date, *MSX1* mutations have been identified in several unrelated families with selective tooth agenesis and selective tooth agenesis in combination with nonsyndromic orofacial clefting and nail abnormalities (Witkop syndrome), respectively, and isolated clefting (Figure 4.5, Figure 4.6, Table 4.2) (Vastardis et al., 1996; van den Boogaard et al., 2000, Jumlongras et al., 2001; Lidral and Reising, 2002; De Muynck et al., 2004; Suzuki et al., 2004; Vieira et al., 2005; Kim et al., 2006; Mostowska et al., 2006) (Table 4.3, Figure 4.6).

The homeobox gene *Msx1* is highly conserved and therefore expected to play an important role in embryonic development. *Msx1* protein has been found in regions of cephalic neural crest migration and differentiation, as well as in the derived mesenchyme cells (Hill et al., 1989; Robert et al., 1989; MacKenzie et al., 1991a,b; reviewed by Davidson and Hill, 1991; Davidson 1995; Sadler and Potts, 1997; Houzelstein et al., 1997, 1999, 2000; Bendall and Abate-Shen, 2000; Ramos and Robert, 2005).

Msx1 is also involved in epithelial–mesenchymal interaction by mediating and controlling the expression of inductive signals transmitted between the epithelial and mesenchymal layers during tooth development, palatal development and development of the mandibular arch and limb buds (Davidson, 1995; Thesleff and Sharpe, 1997; Francis-West et al.,

1998; reviewed in Peters and Balling, 1999; Zhang et al., 1999; Bei et al., 2000; reviewed in Bendall and Abate-Shen, 2000; Zhao et al., 2000; Mina, 2001a,b; Zhang et al., 2002; Yamashiro et al 2003; Zhang et al., 2003a; Liu et al., 2005, Levi et al., 2006; Ogawa et al., 2006).

The main function of *Msx1* is to maintain the proliferative capacity of precursor cells of the mesenchyme by preventing their differentiation. Premature differentiation results in decreased proliferation and, thus, impaired outgrowth and morphogenesis. In addition, *Msx1* is involved in programmed cell death, which also contributes to morphogenesis (Marazzi et al., 1997; Tríbulo et al., 2004).

MSX1 regulates cellular differentiation by transcriptional repression and protein–protein interaction (Zhang et al., 1997, 1999; Bendall and Abate-Shen, 2000). For example, MSX1 could suppress terminal differentiation in myoblasts and osteoblasts by repression of *MyoD* and *Cbfa1* expression, respectively (Blin-Wakkach et al., 2001; Orestes-Cardosa et al. (2002); Zhang et al., 2003a; Cirillo and Zaret 2004; Lee et al., 2004). Hu et al. (2001) proposed that Msx1 gene expression also maintains cyclin D1 expression and prevents cells from exiting the cell cycle, thereby inhibiting terminal differentiation of precursor cells. Han et al. (2003a) supported this model by demonstrating that an *Msx1* null mutation results in significantly elevated expression of the CDK inhibitor $p19^{INK4d}$. In the absence of MSX1, cranial neural crest-derived mesenchyme consequently fails to proliferate and misdifferentiates (Han et al., 2003a). During embryogenesis, *Msx1* is broadly expressed. There was obvious expression in the craniofacial structures, including facial processes, tooth buds, and limb buds. Thus, mutations in *Msx1* were expected to be associated with abnormal morphogenesis of these structures (Hill et al., 1989; Robert et al., 1989; MacKenzie et al., 1991a,b; reviewed by Davidson and Hill, 1991; Davidson 1995; Sadler and Potts, 1997; Houzelstein et al., 1997, 1999; Bendall and Abate-Shen, 2000; Ramos and Robert, 2005).

The phenotypic consequences of lacking *Msx1* function were examined in homozygous $Msx1^{-/-}$ mice. All $Msx1^{-/-}$ mice exhibit cleft secondary palate, deficiency of alveolar mandible and maxilla, and failure of tooth development. Additionally, they manifest abnormalities of the skull, malleus, nasal bones, conchae, and nail plates (Satokata and Maas, 1994; Houzelstein et al., 1997; Jumlongras et al., 2001). Several authors have suggested that the apparent absence of abnormalities in other tissues expressing *Msx1* could be an effect of functional redundancy of *Msx2* (Satokata and Maas, 1994; Catron et al., 1996; Houzelstein et al., 1997). The expression patterns of *Msx1* and *Msx2* overlap considerably, although they are not identical..

Another possibility is that other cofactors are necessary for the proper functioning of

MSX1, and *MSX1* is nonfunctional in sites where the cofactor is not expressed or absent (Houzelstein et al., 1997).

The phenotypic spectrum in the families with an *MSX1* mutation shows a clear resemblance to the phenotype seen in knockout mice. The phenotype is in most cases probably due to haploinsufficiency. In these cases the mutation will affect the homeodomain, which is essential for DNA binding, transcriptional repression, protein–protein interaction, and protein stability of MSX1. MSX1 regulates cellular differentiation with these mechanisms. It is interesting that the mutations that did not disrupt the homeodomain are located within a region that is 100% conserved at the amino acid level across primates and mice (studied by Perry et al., 2006). We cannot rule out that other mechanisms may be at work in addition to, or instead of, haploinsufficiency in causing the different phenotypes seen in humans with an *MSX1* mutation. In this respect, sense/AS mechanism or modification of MSX1 by the small ubiquitin-related modifier (SUMO) may play a role. Until the function of the Msx1 protein and regulation of the gene expression have been further investigated and the effects of the mutations are better understood, we cannot determine a definite pathogenetic mechanism for the deformities so far associated with these mutations.

Acknowledgements

We thank D. Lindhout, J.K. Ploos van Amstel, F.A. Beemer, F. Meijlink, and J. Senior for reading the manuscript and M.J.H.M. van den Boogaard for creating Figure 4.4 and 4.6. Dr. Robert, Pasteur Institute, Paris, kindly provided Figures 4.1, 4.2, and 4.3.

References

1. Akimenko MA, Johnson SL, Westerfield M, Ekker M. Differential induction of four msx homeobox genes during fin development and regeneration in zebra fish. Development 1995;121:347–357.
2. Alappat S, Zhang Z Y, Chen Y P. Msx homeobox gene family and craniofacial development. Cell Res 2003;13:429-442.
3. Bach A, Lallemand Y, Nicola MA, Ramos C, Mathis L, Maufras M, Robert B. Msx1 is required for dorsal diencephalon patterning. Development 2003;130:4025-4036.
4. Beaty TH, Wang H, Hetmanski JB, Fan YT, Zeiger JS, Liang KY, Chiu YF, Vanderkolk CA, Seifert KC, Wulfsberg EA, et al. A case-control study of nonsyndromic oral clefts in Maryland. Ann Epidemiol 2001;11:434–442.
5. Beaty TH, Hetmanski JB, Zeiger JS, Fan YT, Liang KY, Vanderkolk CA, McIntosh I. Testing candidate genes for non-syndromic oral clefts using a case-parent trio design. Genet Epidemiol 2002;22:1–11.
6. Beck CW, Christen B, Slack JM. Molecular pathways needed for regeneration of spinal cord and muscle in a vertebrate. Dev Cell. 2003;5:429-439.
7. Bei M, Maas R. FGFs and BMP4 induce both Msx1-independent and Msx1-dependent signaling pathways in early tooth development. Development 1998;125:4325–4333.
8. Bei M, Kratochwil K, Maas RL. BMP4 rescues a non-cell-autonomous function of Msx1 in tooth development. Development 2000;127:4711–4718.
9. Bell JR, Noveen A, Liu YH, Ma L, Dobias S, Kundu R, Luo W, Xia Y, Lusis AJ, Snead ML. Genomic structure, chromosomal location, and evolution of the mouse Hox8 gene. Genomics 1993;17:800.
10. Bendall AJ, Abate-Shen C. Roles for Msx and Dlx homeoproteins in vertebrate development. Gene 2000;247:17–31.
11. Bendall AJ, Rincon-Limas DE, Botas J, Abate-Shen C. Protein complex formation between Msx1 and Lhx2 homeoproteins is incompatible with DNA binding activity. Differentiation 1998;63:151–157.
12. Bendall AJ, Ding J, Hu G, Shen MM, Abate-Shen C. Msx1 antagonizes the myogenic activity of Pax3 in migrating limb muscle precursors. Development 1998;126:4965–4976.
13. Berdal A, Lezot F, Pibouin L, Hotton D, Ghoul-Mazgar S, Teillaud C, Robert B, MacDougall M, Blin C . Msx1 homeogene antisense mRNA in mouse dental and bone cells. Connect Tissue Res 2002;43:148-152.
14. Blanco R, Chakraborty R, Barton SA, Carreno H, Paredes M, Jara L, Palomino H, Schull WJ. Evidence of a sex-dependent association between the Msx1 locus and nonsyndromic cleft lip with or without cleft palate in the Chilean population. Hum Biol 2001;73:81–89.
15. Blin-Wakkach C, Lezot F, Ghoul-Mazgar S, Hotton D, Monteiro S, Teillaud C, Pibouin L, Orestes-Cardoso S, Papagerakis P, Macdougall M, et al. Endogenous Msx1 antisense transcript: in vivo and in vitro evidences, structure, and potential involvement in skeleton development in mammals. Proc Natl Acad Sci USA 2001;98:7336–7341.
16. Brown JM, Wedden SE, Millburn GH, Robson LG, Hill RE, Davidson DR, Tickle C. Experimental analysis of the control of expression of the homeobox-gene Msx-1 in the developing limb and face. Development 1993;119:41–48.
17. Carlson MR, Bryant SV, Gardiner DM. Expression of Msx-2 during development, regeneration, and wound healing in axolotl limbs. J Exp Zool 1998;282:715–723.

18. Catron KM, Iler N, Abate C. Nucleotides flanking a conserved TAAT core dictate the DNA binding specificity of three murine homeodomain proteins. Mol Cell Biol 1993;13:2354–2365.
19. Catron KM, Zhang H, Marshall SC, Inostroza JA, Wilson JM, Abate C. Transcriptional repression by Msx-1 does not require homeodomain DNA-binding sites. Mol Cell Biol 1995;15:861–871.
20. Catron KM, Wang H, Hu G, Shen MM, Abate-Shen C (1996). Comparison of MSX-1 and MSX-2 suggests a molecular basis for functional redundancy. Mech Dev 1996;55:185–199.
21. Celli J, Duijf P, Hamel BC, Bamshad M, Kramer B, Smits AP, Newbury-Ecob R, Hennekam RC, Van Buggenhout G, Van Haeringen A, et al. Heterozygous germline mutations in the p53 homolog p63 are the cause of EEC syndrome. Cell 1999;99: 143–153
22. Chan-Thomas PS, Thompson RP, Robert B, Yacoub MH, Barton PJ. Expression of homeobox genes Msx-1 (Hox-7) and Msx-2 (Hox-8) during cardiac development in the chick. Dev Dyn 1993;197:203–216.
23. Chen Y, Bei M, Woo I, Satokata I, Maas R. Msx1 controls inductive signaling in mammalian tooth morphogenesis. Development 1996;122:3035–3044.
24. Chitty LS, Dennis N, Baraitser M. Hidrotic ectodermal dysplasia of hair, teeth, and nails: case reports and review. J Med Genet 1996;33:707–710.
25. Christensen K, Mitchell LE. Familial recurrence-pattern analysis of nonsyndromic isolated cleft palate—a Danish Registry study. Am J Hum Genet 1996;58:182–190.
26. Cirillo L, Zaret K. Developmental biology. A linker histone restricts muscle development. Science. 2004;304:1607-1609.
27. Coelho CN, Sumoy L, Rodgers BJ, Davidson DR, Hill RE, Upholt WB, Kosher RA. Expression of the chicken homeobox-containing gene GHox-8 during embryonic chick limb development. Mech Dev 1991a;34: 143–154.
28. Coelho CN, Krabbenhoft KM, Upholt WB, Fallon JF, Kosher RA. Altered expression of the chicken homeobox-containing genes GHox-7 and GHox-8 in the limb buds of limbless mutant chick embryos. Development 1991b;113:1487–1493.
29. Cohen MM Jr. Craniofacial disorders caused by mutations in homeobox genes MSX1 and MSX2. J Craniofac Genet Dev Biol 2000;20:19–25.
30. Cohn MJ, Tickle C. Limbs: a model for pattern formation within the vertebrate body plan. Trends Genet 1996;12:253–257.
31. Coudert AE, Pibouin L, Vi-Fane B, Thomas BL, Macdougall M, Choudhury A, Robert B, Sharpe PT, Berdal A, Lezot F. Expression and regulation of the Msx1 natural antisense transcript during development. Nucleic Acids Res 2005;33:5208-5218.
32. Cox T C. Taking it to the max: The genetic and developmental mechanisms coordinating midfacial morphogenesis and dysmorphology. Clin Genet 2004;65: 163-176.
33. Crews L, Gates PB, Brown R, Joliot A, Foley C, Brockes JP, Gann AA (1995). Expression and activity of the newt Msx-1 gene in relation to limb regeneration. Proc R Soc Lond B Biol Sci 259: 161–171.
34. Davidson D. The function and evolution of Msx genes: pointers and paradoxes. Trends Genet 1995;11: 405–411.
35. Davidson DR, Hill RE. Msh-like genes: family of homeobox genes with wide-ranging expression during vertebrate development. Semin Dev Biol 1991;2: 405–412.
36. Davidson DR, Crawley A, Hill RE, Tickle C. Position-dependent expression of two related homeobox genes in developing vertebrate limbs. Nature 1991;352: 429–431.

37. De Muynck S, Schollen E, Matthijs G, Verdonck A, Devriendt K, Carels C. A novel MSX1 mutation in hypodontia. Am J Med Genet 2004;128A: 401-3.
38. Ekker M, Akimenko MA, Bremiller R, Westerfield M. Regional expression of three homeobox transcripts in the inner ear of zebra fish embryos. Neuron 1992;9: 27–35.
39. Fallin MD, Hetmanski JB, Park J, Scott AF, Ingersoll R, Fuernkranz HA, McIntosh I, Beaty TH. Family-based analysis of MSX1 haplotypes for association with oral clefts. Genet Epidemiol. 2003;25: 168-175.
40. Ferguson MW. A hole in the head. Nat Genet 2000;24: 330–331.
41. Fernandez-Llebrez P, Grondona JM, Perez J, Lopez-Aranda MF, Estivill-Torrus G, Llebrez-Zayas PF, Soriano E, Ramos C, Lallemand Y, Bach A, Robert B. Msx1-deficient mice fail to form prosomere 1 derivatives, subcommissural organ, and posterior commissure and develop hydrocephalus. J Neuropathol Exp Neurol 2004;63: 574-586.
42. FitzPatrick D, Farrall M. An estimation of the number of susceptibility loci for isolated cleft palate. J Craniofac Genet Dev Biol 1993;13: 230–235.
43. Francis-West P, Ladher R, Barlow A, Graveson A. Signalling interactions during facial development. Mech Dev 1998;75: 3–28.
44. Fraser FC. Thoughts on the etiology of clefts of the palate and lip. Acta Genet 1955;5: 358–369.
45. Fraser FC. The genetics of cleft lip and cleft palate. Am J Hum Genet 1970;22: 336–352.
46. Friedmann Y, Daniel CW. Regulated expression of homeobox genes Msx-1 and Msx-2 in mouse mammary gland development suggests a role in hormone action and epithelial–stromal interactions. Dev Biol 1996;177: 347–355.
47. Garzon MC, Paller AS. What syndrome is this? Witkop tooth and nail syndrome. Pediatr Dermatol 1996;13: 63–64.
48. Gersch RP, Lombardo F, McGovern SC, Hadjiargyrou M. Reactivation of Hox gene expression during bone regeneration. J Orthop Res. 2005;23:882-890.
49. Giansanti JS, Long SM, Rankin JL. The "tooth and nail" type of autosomal dominant ectodermal dysplasia. Oral Surg Oral Med Oral Pathol 1974;37: 576–582.
50. Graber LW. Congenital absence of teeth: a review with emphasis on inheritance patterns. J Am Dent Assoc 1978;96: 266–275.
51. Gupta V, Bei M. Modification of Msx1 by SUMO-1. Biochem Biophys Res Commun 2006;345:74-77.
52. Han J, Ito Y, Yeo JY, Sucov HM, Maas R, Chai Y. Cranial neural crest-derived mesenchymal proliferation is regulated by Msx1-mediated p19(INK4d) expression during odontogenesis. Dev Biol 2003a;26:183-196.
53. Han M, Yang X, Farrington JE, Muneoka K. Digit regeneration is regulated by Msx1 and BMP4 in fetal mice. Development 2003b;130: 5123-5132.
54. Han M, Yang X, Taylor G, Burdsal CA, Anderson RA, Muneoka K. Limb regeneration in higher vertebrates: developing a roadmap. Anat Rec B New Anat 2005;287: 14-24.
55. Hewitt JE, Clark LN, Ivens A, Williamson R. Structure and sequence of the human homeobox gene HOX7. Genomics 1991;11: 670–678.
56. Hill RE, Jones PF, Rees AR, Sime CM, Justice MJ, Copeland NG, Jenkins NA, Graham E, Davidson DR. A new family of mouse homeo box-containing genes: molecular structure, chromosomal location, and developmental expression of Hox-7.1. Genes Dev 1989;3: 26–37.

57. Hodges SJ, Harley KE. Witkop tooth and nail syndrome: report of two cases in a family. Int J Paediatr Dent 1999;9: 207–211.

58. Holland PW. Cloning and evolutionary analysis of msh-like homeobox genes from mouse, zebrafish and ascidian. Gene 1991;98: 253–257.

59. Houzelstein D, Cohen A, Buckingham ME, Robert B. Insertional mutation of the mouse Msx1 homeobox gene by an nlacZ reporter gene. Mech Dev 1997;65: 123–133.

60. Houzelstein D, Auda-Boucher G, Cheraud Y, Rouaud T, Blanc I, Tajbakhsh S, Buckingham ME, Fontaine-Perus J, Robert B. The homeobox gene Msx1 is expressed in a subset of somites, and in muscle progenitor cells migrating into the forelimb. Development 1999;126: 2689–2701.

61. Houzelstein D, Cheraud Y, Auda-Boucher G, Fontaine-Perus J, Robert B. The expression of the homeobox gene Msx1 reveals two populations of dermal progenitor cells originating from the somites. Development 2000;127: 2155–2164.

62. Hu G, Vastardis H, Bendall AJ, Wang Z, Logan M, Zhang H, Nelson C, Stein S, Greenfield N, Seidman CE, et al. Haploinsufficiency of Msx1: a mechanism for selective tooth agenesis. Mol Cell Biol 1998;18: 6044–6051.

63. Hu G, Lee H, Price SM, Shen MM, Abate-Shen C. Msx homeobox genes inhibit differentiation through upregulation of cyclin D1. Development 2001;128: 2373–2384.

64. Hudson CD, Witkop CJ. Autosomal dominant hypodontia with nail dysgenesis. Report of twenty-nine cases in six families. Oral Surg Oral Med Oral Pathol 1975;39: 409–423.

65. Inouye M. Antisense RNA: its functions and applications in gene regulation—a review. Gene 1988;72: 25–34.

66. Isaac VE, Sciavolino P, Abate C. Multiple amino acids determine the DNA binding specificity of the Msx-1 homeodomain. Biochemistry 1995;34: 7127–7134.

67. Ishii M, Han J, Yen HY, Sucov HM, Chai Y, Maxson RE Jr. Combined deficiencies of Msx1 and Msx2 cause impaired patterning and survival of the cranial neural crest. Development 2005;132: 4937-4950.

68. Ivens A, Flavin N, Williamson R, Dixon M, Bates G, Buckingham M, Robert B. The human homeobox gene HOX7 maps to chromosome 4p16.1 and may be implicated in Wolf-Hirschhorn syndrome. Hum Genet 1990;84: 473–476.

69. Jabs EW, Muller U, Li X, Ma L, Luo W, Haworth IS, Klisak I, Sparkes R, Warman ML, Mulliken JB. A mutation in the homeodomain of the human MSX2 gene in a family affected with autosomal dominant craniosynostosis. Cell 1993;75: 443–450.

70. Jezewski PA, Vieira AR, Nishimura C, Ludwig B, Johnson M, O'Brien SE, Daack-Hirsch S, Schultz RE, Weber A, Nepomucena B, Romitti PA, Christensen K, Orioli IM, Castilla EE, Machida J, Natsume N, Murray JC. Complete sequencing shows a role for MSX1 in non-syndromic cleft lip and palate. J Med Genet 2003;40: 399-407.

71. Johnson RL, Tabin CJ. Molecular models for vertebrate limb development. Cell 1997;90: 979–990.

72. Jowett AK, Vainio S, Ferguson MW, Sharpe PT, Thesleff I. Epithelial-mesenchymal interactions are required for msx 1 and msx 2 gene expression in the developing murine molar tooth. Development 1993;117: 461–470.

73. Jugessur A, Lie RT, Wilcox AJ, Murray JC, Taylor JA, Saugstad OD, Vindenes HA, Abyholm F. Variants of developmental genes (TGFA, TGFB3, and MSX1) and their associations with orofacial clefts: a case-parent triad analysis. Genet Epidemiol 2003;24: 230-239.

74. Jumlongras D, Bei M, Stimson JM, Wang WF, DePalma SR, Seidman CE, Felbor U, Maas R, Seidman JG, Olsen BR. A nonsense mutation in Msx1 causes Witkop syndrome. Am J Hum Genet 2001;69: 67–74.
75. Kim HJ, Rice DP, Kettunen PJ, Thesleff I. FGF-, BMP- and Shh-mediated signalling pathways in the regulation of cranial suture morphogenesis and calvarial bone development. Development 1998;125: 1241–1251.
76. Kim JW, Simmer JP, Lin BP, Hu JC. Novel MSX1 Frameshift Causes Autosomal-dominant Oligodontia. J Dent Res 2006;85: 267-271.
77. Kondo S, Schutte B, Richardson R, Bjork B, Knight A, Watanabe Y, Howard E, Ferreira de Lima R, Daack-Hirsch S, Sander A, et al. Mutations in IRF6 cause Van der Woude and popliteal pterygium syndromes. Nat Genet 2002;32: 285–289.
78. Koshiba K, Kuroiwa A, Yamamoto H, Tamura K, Ide H. Expression of Msx genes in regenerating and developing limbs of axolotl. J Exp Zool 1998;282: 703–714.
79. Kumar M, Carmichael GG. Antisense RNA: function and fate of duplex RNA in cells of higher eukaryotes. Microbiol Mol Biol Rev 1998;62: 1415–1434.
80. Kumar A, Velloso CP, Imokawa Y, Brockes JP. The regenerative plasticity of isolated urodele myofibers and its dependence on MSX1. PLoS Biol. 2004;2: E218.
81. Lallemand Y, Nicola MA, Ramos C, Bach A, Cloment CS, Robert B. Analysis of Msx1; Msx2 double mutants reveals multiple roles for Msx genes in limb development. Development 2005;132: 3003-3014.
82. Lee TI, Young RA. Regulation of gene expression by TBP-associated proteins. Genes Dev 1998;12: 1398–1408.
83. Lee H, Habas R, Abate-Shen C. MSX1 cooperates with histone H1b for inhibition of transcription and myogenesis. Science 2004;304: 1675-1678.
84. Lee H, Quinn JC, Prasanth KV, Swiss VA, Economides KD, Camacho MM, Spector DL, Abate-Shen C. PIAS1 confers DNA-binding specificity on the Msx1 homeoprotein. Genes Dev 2006;20: 784-794.
85. Levi G, Mantero S, Barbieri O, Cantatore D, Paleari L, Beverdam A, Genova F, Robert B, Merlo GR. Msx1 and Dlx5 act independently in development of craniofacial skeleton, but converge on the regulation of Bmp signaling in palate formation. Mech Dev 2006;123: 3-16.
86. Lidral AC, Reising BC. The role of MSX1 in human tooth agenesis. J Dent Res 2002;81: 274-8
87. Lidral AC, Romitti PA, Basart AM, Doetschman T, Leysens NJ, Daack-Hirsch S, Semina EV, Johnson LR, Machida J, Burds A, et al. Association of Msx1 and TGFB3 with nonsyndromic clefting in humans. Am J Hum Genet 1998;63: 557–568.
88. Line SR. Variation of tooth number in mammalian dentition: connecting genetics, development, and evolution. Evol Dev 2003;5: 295-304.
89. Liu Y, Helms A W, Johnson J E. Distinct activities of Msx1 and Msx3 in dorsal neural tube development. Development 2004;131: 1017-1028.
90. Liu W, Sun X, Braut A, Mishina Y, Behringer RR, Mina M, Martin JF. Distinct functions for Bmp signaling in lip and palate fusion in mice. Development 2005;132: 1453-61.
91. Lu CH, Rincon-Limas DE, Botas J. Conserved overlapping and reciprocal expression of msh/Msx1 and apterous/Lhx2 in Drosophila and mice. Mech Dev 2000;99:177–181.
92. Lumsden AG. Spatial organization of the epithelium and the role of neural crest cells in the initiation of the mammalian tooth germ. Development 1988;103(Suppl): 155–169.

93. Lyons GE, Houzelstein D, Sassoon D, Robert B, Buckingham ME. Multiple sites of Hox-7 expression during mouse embryogenesis: comparison with retinoic acid receptor mRNA localization. Mol Reprod Dev 1992;32: 303–314.

94. Maas R, Bei M. The genetic control of early tooth development. Crit Rev Oral Biol Med 1997;8: 4–39.

95. Maas R, Chen YP, Bei M, Woo I, Satokata I. The role of Msx genes in mammalian development. Ann NY Acad Sci 1996;785: 171–181.

96. MacKenzie A, Ferguson MW, Sharpe PT. Hox-7 expression during murine craniofacial development. Development 1991a;113: 601–611.

97. MacKenzie A, Leeming GL, Jowett AK, Ferguson MW, Sharpe PT. The homeobox gene Hox 7.1 has specific regional and temporal expression patterns during early murine craniofacial embryogenesis, especially tooth development in vivo and in vitro. Development 1991b;111: 269–285.

98. MacKenzie A, Ferguson MW, Sharpe PT. Expression patterns of the homeobox gene, Hox-8, in the mouse embryo suggest a role in specifying tooth initiation and shape. Development 1992;115: 403–420.

99. Marazita ML, Murray JC, Lidral AC, Arcos-Burgos M, Cooper ME, Goldstein T, Maher BS, Daack-Hirsch S, Schultz R, Mansilla MA, Field LL, Liu YE, Prescott N, Malcolm S, Winter R, Ray A, Moreno L, Valencia C, Neiswanger K, Wyszynski DF, Bailey-Wilson JE, Albacha-Hejazi H, Beaty TH, McIntosh I, Hetmanski JB, Tuncbilek G, Edwards M, Harkin L, Scott R, Roddick LG. Meta-analysis of 13 genome scans reveals multiple cleft lip/palate genes with novel loci on 9q21 and 2q32-35. Am J Hum Genet 2004;75: 161-173.

100. Marazzi G, Wang Y, Sassoon D. Msx2 is a transcriptional regulator in the BMP4-mediated programmed cell death pathway. Dev Biol 1997;186: 127–138.

101. McGrath JA, Duijf PHG, Doetsch V, Irvine AD, De Waal R, Vanmolkot KRJ, Kelly A, Atherton DJ, Griffiths AD, Orlow SJ, et al. Hay-Wells syndrome is caused by heterozygous missense mutations in the SAM domain of p63. Hum Mol Genet 2001;10: 221–229.

102. Milan M, Weihe U, Tiong S, Bender W, Cohen SM. msh specifies dorsal cell fate in the Drosophila wing. Development 2001;128: 3263–3268.

103. Mina M. Morphogenesis of the medial region of the developing mandible is regulated by multiple signaling pathways. Cells Tissues Organs 2001a;169: 295–301.

104. Mina M Regulation of mandibular growth and morphogenesis. Crit Rev Oral Biol Med 2001b;12: 276–300.

105. Mina M, Kollar EJ. The induction of odontogenesis in non-dental mesenchyme combined with early murine mandibular arch epithelium. Arch Oral Biol 1987;32: 123–127.

106. Mina M, Gluhak J, Upholt WB, Kollar EG, Rogers B. Experimental analysis of Msx1 and Msx2 gene expression during chick mandibular morphogenesis. Dev Dyn 1995;202: 195–214.

107. Modesto A, Moreno LM, Krahn K, King S, Lidral AC. MSX1 and orofacial clefting with and without tooth agenesis. J Dent Res 2006;85: 542-546.

108. Monaghan AP, Davidson DR, Sime C, Graham E, Baldock R, Bhattacharya SS, Hill RE. The Msh-like homeobox genes define domains in the developing vertebrate eye. Development 1991;112: 1053–1061.

109. Moreno LM, Arcos-Burgos M, Marazita ML, Krahn K, Maher BS, Cooper ME, Valencia-Ramirez CR, Lidral AC. Genetic analysis of candidate loci in non-syndromic cleft lip families from Antioquia-Colombia and Ohio. Am J Med Genet 2004;125A: 135-144.

110. Mostowska A, Biedziak B, Trzeciak WH. A novel c.581C>T transition localized in a highly conserved homeobox sequence of MSX1: is it responsible for oligodontia? J Appl Genet 2006;47: 159-64.

111. Murciano C, Fernandez TD, Duran I, Maseda D, Ruiz-Sanchez J, Becerra J, Akimenko MA, Mari-Beffa M. Ray-interray interactions during fin regeneration of Danio rerio. Dev Biol 2002;252: 214-224.

112. Murdoch-Kinch CA, Miles DA, Poon CK. Hypodontia and nail dysplasia syndrome. Report of a case. Oral Surg Oral Med Oral Pathol 1993;75: 403–406.

113. Murray JC. Gene/environment causes of cleft lip and/or palate. Clin Genet 2002;61: 248–256.

114. Nechiporuk A, Keating MTA. Proliferation gradient between proximal and msxb-expressing distal blastema directs zebrafish fin regeneration. Development 2002;129: 2607-2617 .

115. Nieminen P, Arte S, Pirinen S, Peltonen L, Thesleff I. Gene defect in hypodontia: exclusion of MSX1 and MSX2 as candidate genes. Hum Genet 1995;96: 305–308.

116. Nohno T, Noji S, Koyama E, Nishikawa K, Myokai F, Saito T, Taniguchi S. Differential expression of two msh-related homeobox genes Chox-7 and Chox-8 during chick limb development. Biochem Biophys Res Commun 1992;182: 121–128.

117. Noveen A, Jiang TX, Ting-Berreth SA, Chuong CM. Homeobox genes Msx-1 and Msx-2 are associated with induction and growth of skin appendages. J Invest Dermatol 1995;104: 711–719.

118. Odelberg SJ, Kollhoff A, Keating MT. Dedifferentiation of mammalian myotubes induced by msx1. Cell 2000;103: 1099–1109.

119. Ogawa T, Kapadia H, Feng J Q, Raghow Rajendra, Peters H, D'Souza R N. Functional Consequences of Interactions between Pax9 and Msx1 Genes in Normal and Abnormal Tooth Development. J Biol Chem. 2006;281: 18363-18369.

120. Ogi H, Suzuki K, Ogino Y, Kamimura M, Miyado M, Ying X, Zhang Z, Shinohara M, Chen Y, Yamada G. Ventral abdominal wall dysmorphogenesis of Msx1/Msx2 double-mutant mice. Anat Rec A Discov Mol Cell Evol Biol 2005;284: 424-30

121. Orestes-Cardoso SM, Nefussi JR, Hotton D, Mesbah M, Orestes-Cardoso MD, Robert B, Berdal A. Postnatal Msx1 expression pattern in craniofacial, axial, and appendicular skeleton of transgenic mice from the first week until the second year. Dev Dyn 2001;221: 1-13.

122. Orestes-Cardoso S, Nefussi JR, Lezot F, Oboeuf M, Pereira M, Mesbah M, Robert B, Berdal A. Msx1 is a regulator of bone formation during development and postnatal growth: in vivo investigations in a transgenic mouse model. Connect Tissue Res 2002;43: 153-60.

123. Padanilam BJ, Stadler HS, Mills KA, McLeod LB, Solursh M, Lee B, Ramirez F, Buetow KH, Murray JC. Characterization of the human HOX 7 cDNA and identification of polymorphic markers. Hum Mol Genet 1992;1: 407–410.

124. Park J, Park K, Kim S, Lee JH. Msx1 gene overexpression induces G1 phase cell arrest in human ovarian cancer cell line OVCAR3. Biochem Biophys Res Commun 2001;281: 1234–1240.

125. Park K, Kim K, Rho SB, Choi K, Kim D, Oh SH, Park J, Lee SH, Lee JH (2005). Homeobox Msx1 interacts with p53 tumor suppressor and inhibits tumor growth by inducing apoptosis. Cancer Res 65: 749-757.

126. Pavlova A, Boutin E, Cunha G, Sassoon D. Msx1 (Hox-7.1) in the adult mouse uterus: cellular interactions underlying regulation of expression. Development 1994;120: 335–345.

127. Perry GH, Verrelli BC, Stone AC. Comparative analyses reveal a complex history of molecular evolution for human MYH16. Mol Biol Evol. 2005;22:379-82. Erratum in: Mol Biol vol. 2005;22:1156.

128. Perry GH, Verrelli BC, Stone AC. Molecular Evolution of the primate developmental genes MSX1 and PAX9. Mol Biol Evol 2006;23: 644-654.

129. Peters H, Balling R. Teeth. Where and how to make them. Trends Genet 1999;15: 59–65.

130. Phippard DJ, Weber-Hall SJ, Sharpe PT, Naylor MS, Jayatalake H, Maas R, Woo I, Roberts-Clark D, Francis-West PH, Liu YH, et al. Regulation of Msx-1, Msx-2, Bmp-2 and Bmp-4 during foetal and postnatal mammary gland development. Development 1996;122: 2729–2737.

131. Ramos C, Fernandez-Llebrez P, Bach A, Robert B, Soriano E. Msx1 disruption leads to diencephalon defects and hydrocephalus. Dev Dyn 2004;230: 446-460.

132. Ramos C and Robert B. Msh/Msx gene family in neural development. Trends in Genet 2005;21: 624-632.

133. Raya A, Koth CM, Buscher D, Kawakami Y, Itoh T, Raya RM, Sternik G, Tsai HJ, Rodriguez-Esteban C, Izpisua-Belmonte JC. Activation of Notch signaling pathway precedes heart regeneration in zebrafish. Proc Natl Acad Sci U S A. 2003;100: 11889-11895.

134. Ranta R. A review of tooth formation in children with cleft lip/palate. Am J Orthod Dentofacial Orthop 1986;90: 11–18.

135. Redpath TH, Winter GB. Autosomal dominant ectodermal dysplasia with significant dental defects. Br Dent J 1969;126: 123–128.

136. Reginelli AD, Wang YQ, Sassoon D, Muneoka K. Digit tip regeneration correlates with regions of Msx1 (Hox 7) expression in fetal and newborn mice. Development 1995;121: 1065–1076.

137. Richman JM, Tickle C. Epithelia are interchangeable between facial primordial of chick embryos and morphogenesis is controlled in the mesenchyme. Dev Biol 1989;136: 201–210

138. Robert B, Sassoon D, Jacq B, Gehring W, Buckingham M. Hox-7, a mouse homeobox gene with a novel pattern of expression during embryogenesis. EMBO J 1989;8: 91–100.

139. Robert B, Lyons G, Simandl BK, Kuroiwa A, Buckingham M. The apical ectodermal ridge regulates Hox-7 and Hox-8 gene expression in developing chick limb buds. Genes Dev 1991;5: 2363–2374.

140. Romitti PA, Lidral AC, Munger RG, Daack-Hirsch S, Burns TL, Murray JC. Candidate genes for nonsyndromic cleft lip and palate and maternal cigarette smoking and alcohol consumption: evaluation of genotype-environment interactions from a population-based case-control study of orofacial clefts. Teratology 1999;59: 39–50.

141. Sadler TW, Potts LF (1997). Making sense with anti-sense; determining the role of Msx genes in mouse whole embryo culture. In: Studies in Somatology and Craniofacial Biology. Cohen MM Jr, Baum BJ (eds.) IOS Press, Amsterdam, pp. 59–66.

142. Satokata I, Maas R. Msx1 deficient mice exhibit cleft palate and abnormalities of craniofacial and tooth development. Nat Genet 1994;6: 348–356.

143. Schalk-van der Weide Y (1992). Oligodontia: A Clinical, Radiographic and Genetic Evaluation. University of Utrecht, Utrecht, the Netherlands. Dissertation.

144. Schultz RE, Cooper ME, Daack-Hirsch S, Shi M, Nepomucena B, Graf KA, O'Brien EK, O'Brien SE, Marazita ML, Murray JC. Targeted scan of fifteen regions for nonsyndromic cleft lip and palate in Filipino families. Am J Med Genet 2004;125A: 7-22.

145. Schummer M, Scheurlen I, Schaller C, Galliot B. HOM/HOX homeobox genes are present in hydra (Chlorohydra viridissima) and are differentially expressed during regeneration. EMBO J 1992;11: 1815–1823.

146. Schutte BC, Murray JC. The many faces and factors of orofacial clefts. Hum Mol Genet 1999;8: 1853–1859.

147. Scott MP. Vertebrate homeobox gene nomenclature. Cell 1992;71: 551–553.

148. Shang Z, Isaac VE, Li H, Patel L, Catron KM, Curran T, Montelione GT, Abate C. Design of a "minimal" homeodomain: the N-terminal arm modulates DNA binding affinity and stabilizes homeodomain structure. Proc Natl Acad Sci USA 1994;91: 8373–8377.

149. Shetty S, Takahashi T, Matsui H, Ayengar R, Raghow R. Transcriptional autorepression of Msx1 gene is mediated by interactions of Msx1 protein with a multi-protein transcriptional complex containing TATA-binding protein, Sp1 and cAMP-response-element-binding protein-binding protein (CBP/p300). Biochem J 1999;339(Pt 3): 751–758.

150. Shimeld SM, McKay IJ, Sharpe PT. The murine homeobox gene Msx-3 shows highly restricted expression in the developing neural tube. Mech Dev 1996;55: 201–210.

151. Slavkin HC, Shum L, Nuckolls GH (1998). Ectodermal dysplasia: a synthesis between evolutionary, developmental, and molecular biology and human clinical genetics. In: Molecular Basis of Epithelial Appendage Morphogenesis: Molecular Biology Intelligence Unit. Chuong C-M (ed.) RG Landes, Austin, pp. 15–37.

152. Slayton RL, Williams L, Murray JC, Wheeler JJ, Lidral AC, Nishimura CJ. Genetic association studies of cleft lip and/or palate with hypodontia outside the cleft region. Cleft Palate Craniofac J 2003;40: 274-279.

153. Song K, Wang Y, Sassoon D. Expression of Hox-7.1 in myoblasts inhibits terminal differentiation and induces cell transformation. Nature 1992;360: 477–481.

154. Spritz RA. The genetics and epigenetics of orofacial clefts. Curr Opin Pediatr 2001;13: 556–560.

155. Stelnicki EJ, Komuves LG, Holmes D, Clavin W, Harrison MR, Adzick NS, Largman C. The human homeobox genes MSX-1, MSX-2, and MOX-1 are differentially expressed in the dermis and epidermis in fetal and adult skin. Differentiation 1997;62: 33–41.

156. Stimson JM, Sivers JE, Hlava GL. Features of oligodontia in three generations. J Clin Pediatr Dent 1997;21: 269–275.

157. Stock DW, Weiss KM, Zhao Z. Patterning of the mammalian dentition in development and evolution. Bioessays 1997;19: 481–490.

158. Su MW, Suzuki HR, Solursh M, Ramirez F. Progressively restricted expression of a new homeobox-containing gene during Xenopus laevis embryogenesis. Development 1991;111: 1179–1187.

159. Suazo J, Santos JL, Carreno H, Jara L, Blanco R. Linkage disequilibrium between MSX1 and non-syndromic cleft lip/palate in the Chilean population. J Dent Res 2004;83: 782-785.

160. Suzuki HR, Padanilam BJ, Vitale E, Ramirez F, Solursh M. Repeating developmental expression of G-Hox 7, a novel homeobox-containing gene in the chicken. Dev Biol 1991;148: 375–388.

161. Suzuki Y, Jezewski PA, Machida J, Watanabe Y, Shi M, Cooper ME, Viet le T, Nguyen TD, Hai H, Natsume N, Shimozato K, Marazita ML, Murray JC. In a Vietnamese population, MSX1 variants contribute to cleft lip and palate. Genet Med 2004;6: 117-125.

162. Symons AL, Stritzel F, Stamation J. Anomalies associated with hypodontia of the permanent lateral incisor and second premolar. J Clin Pediatr Dent 1993;17: 109–111.

163. Takahashi Y, Le Douarin N. cDNA cloning of a quail homeobox gene and its expression in neural crest–derived mesenchyme and lateral plate mesoderm. Proc Natl Acad Sci USA 1990;87: 7482–7486.

164. Takahashi K, Nuckolls GH, Tanaka O, Semba I, Takahashi I, Dashner R, Shum L, Slavkin HC. Adenovirus-mediated ectopic expression of Msx2 in even-numbered rhombomeres induces apoptotic elimination of cranial neural crest cells in ovo. Development 1998;125: 1627–1635.

165. Thesleff I. Two genes for missing teeth. Nat Genet 1996;13: 379–380.

166. Thesleff I, Sharpe P. Signalling networks regulating dental development. Mech Dev 1997;67: 111–123.

167. Thesleff I, Vaahtokari A, Partanen AM. Regulation of organogenesis. Common molecular mechanisms regulating the development of teeth and other organs. Int J Dev Biol 1995;39: 35–50.

168. Tríbulo C, Aybar M J, Sánchez S S, Mayor R. A balance between the anti-apoptotic activity of Slug and the apoptotic activity of msx1 is required for the proper development of the neural crest. Dev Biol 2004;275: 325-342.

169. Tucker AS, Matthews KL, Sharpe PT. Transformation of tooth type induced by inhibition of BMP signaling. Science 1998;282: 1136–1138.

170. Vainio S, Karavanova I, Jowett A, Thesleff I. Identification of BMP-4 as a signal mediating secondary induction between epithelial and mesenchymal tissues during early tooth development. Cell 1993;75: 45–58.

171. Van den Boogaard MJ, Dorland M, Beemer FA, Ploos van Amstel HK. Msx1 mutation is associated with orofacial clefting and tooth agenesis in humans. Nat Genet 2000;24: 342–343.

172. Vastardis H, Karimbux N, Guthua S W, Seidman JG, Seidman CE. A human Msx1 homeodomain missense mutation causes selective tooth agenesis. Nat Genet 1996;13: 417–421.

173. Vastardis H. The genetics of human tooth agenesis: new discoveries for understanding dental anomalies. Am J Orthod Dentofacial Orthop 2000;117:650-656.

174. Vieira AR, Orioli IM, Castilla EE, Cooper ME, Marazita ML, Murray JC. MSX1 and TGFB3 contribute to clefting in South America. J Dent Res 2003;82: 289-292.

175. Vieira AR, Avila JR, Daack-Hirsch S, Dragan E, Felix TM, Rahimov F, Harrington J, Schultz RR, Watanabe Y, Johnson M, Fang J, O'Brien SE, Orioli IM, Castilla EE, Fitzpatrick DR, Jiang R, Marazita ML, Murray JC. Medical sequencing of candidate genes for nonsyndromic cleft lip and palate. PLoS Genet 2005;1: e64.

176. Walldorf U, Fleig R, Gehring WJ. Comparison of homeobox-containing genes of the honeybee and Drosophila. Proc Natl Acad Sci USA 1989;86: 9971–9975.

177. Wang W, Chen X, Xu H, Lufkin T. Msx3: a novel murine homologue of the Drosophila msh homeobox gene restricted to the dorsal embryonic central nervous system. Mech Dev 1996;58: 203–215.

178. Wang YH, Upholt WB, Sharpe PT, Kollar EJ, Mina M. Odontogenic epithelium induces similar molecular responses in chick and mouse mandibular mesenchyme. Dev Dyn 1998;213: 386–397.

179. Wedden SE. Epithelial–mesenchymal interactions in the development of chick facial primordia and the target of retinoid action. Development1987;99: 341–351.

180. Wedden SE, Ralphs JR, Tickle C. Pattern formation in the facial primordia. Development 1988;103: 31–40.

181. Weech AA. Hereditary ectodermal dysplasia (congenital ectodermal defect). Am J Dis Child 1929;37: 766–790.

182. Wilkie AOM, Tang Z, Elanko N, Walsh S, Twigg SRF, Hurst JA, Wall SA, Chrzanowska, Maxson RE Jr. Functional haploinsufficiency of the human homeobox gene MSX2 casuses defects in skull ossification. Nat Genet 2000;24: 387–390.

183. Witkop CJ Jr (1965). Genetic diseases of the oral cavity. In: Oral pathology. Tiecke RW (ed.) McGraw-Hill, New York, pp. 812–813.

184. Witkop CJ Jr (1990). Hypodontia-nail dysgenesis. In: Birth Defect Encyclopedia: The Comprehensive, Systematic, Illustrated Reference Source for the Diagnosis, Delineation, Etiology, Biodynamics, Occurrence, Prevention, and Treatment of Human Anomalies of Clinical Relevance. Buyse ML (ed.) Center for Birth Defects Information Services, Dover, MA, p. 920.

185. Witkop CJ Jr, Brearley LJ, Gentry WC. Hypoplastic enamel, onycholysis, and hypohidrosis inherited as an autosomal dominant trait: a review of ectodermal dysplasia syndromes. Oral Surg Oral Med Oral Pathol 1975;30: 71–86.

186. Woloshin P, Song K, Degnin C, Killary AM, Goldhamer DJ, Sassoon D, Thayer MJ. MSX1 inhibits myoD expression in fibroblast310T1/2 cell hybrids. Cell 1995;82: 611–620.

187. Yamashiro T, Tummers M, Thesleff I. Expression of bone morphogenetic proteins and Msx genes during root formation J Dent Res 2003;82:172-176.

188. Yokoyama H, Yonei-Tamura S, Endo T, Izpisua Belmonte JC, Tamura K, Ide H. Mesenchyme with fgf-10 expression is responsible for regenerative capacity in Xenopus limb buds. Dev Biol 2000;219: 18-29.

189. Yokouchi Y, Ohsugi K, Sasaki H, Kuroiwa A. Chicken homeobox gene Msx-1: structure, expression in limb buds and effect of retinoic acid. Development 1991;113: 431–444.

190. Zabawski EJ Jr, Cohen JB. Hereditary hypodontia and onychorrhexis of the fingernails and toenail koilonychia: Witkop tooth- and nail syndrome. Dermatol Online J 1999;5: 3.

191. Zhang H, Catron KM, Abate-Shen C. A role for the Msx-1 homeodomain in transcriptional regulation: residues in the N-terminal arm mediate TATA binding protein interaction and transcriptional repression. Proc Natl Acad Sci USA 1996;93: 1764–1769.

192. Zhang H, Hu G, Wang H, Sciavolino P, Iler N, Shen MM, Abate-Shen C. Heterodimerization of Msx and Dlx homeoproteins results in functional antagonism. Mol Cell Biol 1997;17: 2920–2932.

193. Zhang Y, Zhao X, Hu Y, St Amand T, Zhang M, Ramamurthy R, Qiu M, Chen Y. Msx1 is required for the induction of Patched by Sonic hedgehog in the mammalian tooth germ. Dev Dyn 1999;215: 45–53.

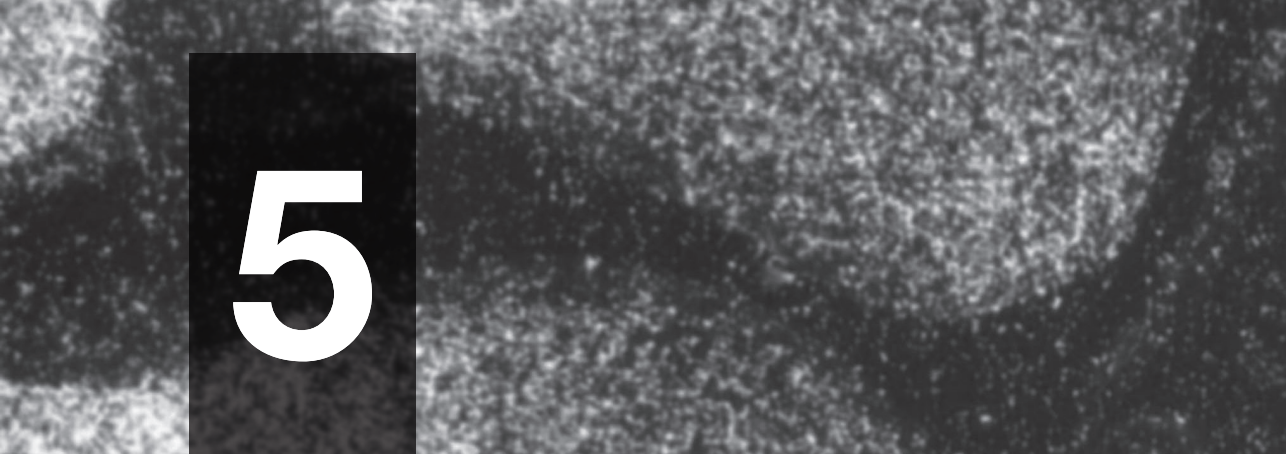
194. Zhang Y, Zhang Z, Zhao X, Yu X, Hu Y, Geronimo B, Fromm SH, Chen YP. A new function of BMP4: dual role for BMP4 in regulation of Sonic hedgehog expression in the mouse tooth germ. Development 2000;127: 1431–1443

195. Zhang Z, Song Y, Zhao X, Zhang X, Fermin C, Chen Y. Rescue of cleft palate in Msx1-deficient mice by transgenic Bmp4 reveals a network of BMP and Shh signaling in the regulation of mammalian palatogenesis. Development. 2002;129:4135-4146.

196. Zhang Z, Song Y, Zhang X, Tang J, Chen J, Chen YP. Msx1/Bmp4 genetic pathway regulates mammalian alveolar bone formation via induction of Dlx5 and Cbfa1. Mech Dev 2003a;120: 1469-1479

197. Zhang Z, Zhang X, Avniel WA, Song Y, Jones SM, Jones TA, Fermin C, Chen Y. Malleal processus brevis is dispensable for normal hearing in mice. Dev Dyn. 2003b;227: 69-77.

198. Zhao X, Zhang Z, Song Y, Zhang X, Zhang Y, Hu Y, Fromm SH, Chen Y. Transgenically ectopic expression of Bmp4 to the Msx1 mutant dental mesenchyme restores downstream gene expression but represses Shh and Bmp2 in the enamel knot of wild type tooth germ. Mech Dev 2000;99:29-38



5

Chapter 5

MSX1 and non-syndromic clefting

The MSX1 allele 4 homozygous child exposed to smoking at periconception is most sensitive in developing nonsyndromic orofacial clefts.

van den Boogaard MJ, de Costa D, Krapels IP, Liu F, van Duijn C, Sinke RJ, Lindhout D, Steegers-Theunissen RP.

Published in: Hum Genet. 2008 Dec;124(5):525-34.

Abstract

Nonsyndromic orofacial clefts (OFC) are common birth defects caused by certain genes interacting with environmental factors. Mutations and association studies indicate that the homeobox gene *MSX1* plays a role in human clefting. In a Dutch case-control triad study (mother, father, and child), we investigated interactions between MSX1 and the parents' periconceptional lifestyle in relation to the risk of OFC in their offspring. We studied 181 case- and 132 control mothers, 155 case- and 121 control fathers, and 176 case- and 146 control children, in which there were 107 case triads and 66 control triads. Univariable and multivariable logistic regression analyses were applied, and odds ratios (OR), 95% confidence intervals (CI) were calculated. Allele 4 of the CA marker in the *MSX1* gene, consisting of nine CA repeats, was the most common allele found in both the case and control triads. Significant interactions were observed between allele 4 homozygosity of the child with maternal smoking (OR 2.7, 95% CI 1.1–6.6) and with smoking by both parents (OR 4.9, 95% CI 1.4–18.0). Allele 4 homozygosity in the mother and smoking showed a risk estimate of OR 3.2 (95% CI 1.1–9.0). If allele 4 homozygous mothers did not take daily folic acid supplements in the recommended periconceptional period, this also increased the risk of OFC for their offspring (OR 2.8, 95% CI 1.1–6.7). Our findings show that, in the Dutch population, periconceptional smoking by both parents interacts with a specific allelic variant of *MSX1* to significantly increase OFC risk for their offspring. Possible underlying mechanisms are discussed.

Introduction

Orofacial clefts (OFC) are common birth defects and include nonsyndromic cleft lip (CL) and/or cleft palate (CP). They have a birth prevalence varying from 1 in 1,500 to 1 in 2,500 in Caucasian populations (Schutte and Murray 1999). These figures also depend on ethnic background, geographic origin, lifestyle factors and socioeconomic status (Jugessur and Murray 2005). OFC are most often classified into cleft lip with or without cleft palate (CL/P) and cleft palate only (CPO). The etiology of OFC is complex, meaning both genetic and environmental factors are involved (Jugessur and Murray 2005; Krapels et al. 2006a; Gritli-Linde 2007; Schliekelman and Slatkin 2002). Gene expression studies and a transgenic knock-out model with cleft phenotype have suggested that *Msx1* is involved in the etiology of clefting (Satokata and Maas 1994; Davidson 1995).

Msx1 is a highly conserved homeobox gene that plays several key roles in epithelial–mesenchymal tissue interactions during craniofacial development. It regulates cellular proliferation, differentiation and cell death, which is important for balanced cell growth and morphogenesis (Bendall and Abate-Shen 2000). Extensive studies in knockout mice have also demonstrated that *Msx1-Bmp* signaling, regulating expression of *Shh*, is essential for palate development (Zhang et al. 2002) and the identification of a *MSX1* stop mutation in a Dutch family with a combination of tooth agenesis and OFC confirmed *MSX1* as a candidate gene for clefting in humans (van den Boogaard et al. 2000). Sequencing- and association studies have indicated a role for *MSX1* in the etiology of nonsyndromic orofacial clefting (Lidral et al. 1998; Beaty et al. 2001; Blanco et al. 2004; Fallin et al. 2003; Jezewski et al. 2003; Jugessur et al. 2003; Vieira et al. 2003; Moreno et al. 2004; Vieira et al. 2005; Modesto et al. 2006; Tongkobpetch et al. 2006; Park et al. 2007), although results published by others question this role (Mitchell et al. 2001; Koillinen et al. 2003; Etheredge et al. 2005).

There is increasing evidence that the environment can substantially modulate genetic effects and various lifestyle factors, such as smoking, a folate-deficient diet, alcohol intake and the use of medication, have been associated with OFC (Jugessur and Murray 2005; Krapels et al. 2006a, b). It is important to realize that the mother determines the intrauterine environment in which the fetus develops. Our aim was to investigate any association between *MSX1*-CA markers in the mother, father, and child with periconceptional smoking, alcohol, and medication use by both parents and maternal folic acid supplementation in a Dutch population.

Materials and methods

Study population

The study population and design were described previously (van Rooij et al. 2002). Briefly, between 1998 and 2000, a case-control triad study was conducted by nine of the largest cleft palate teams in the Netherlands (Amsterdam VU, Arnhem, Groningen, Leeuwarden, Nijmegen, Rotterdam, Tilburg, Utrecht, and Zwolle). We recruited children with a nonsyndromic OFC, both parents, and healthy controls. In each hospital team, the OFC was diagnosed by a clinician according to a standard registration form developed by the Dutch Association for Cleft Palate and Craniofacial Anomalies (Luijsterberg and Vermeij-Keers 1999). The standardized registration was performed when the index child

was approximately 15 months old. Most associated malformations and developmental delays are identified in the first year of life, which is important in selecting cases and controls. The unrelated control children did not have major congenital malformations and were enrolled by friends, acquaintances or neighbors of case parents and through well-baby clinics in and around Nijmegen. (Dutch well-baby clinics provide standard check-ups for all young children's growth and development.) All participants were Dutch Caucasians. DNA was obtained via blood samples or buccal swabs. We defined the periconceptional period for mothers as 3 months before conception to 3 months afterward and for the fathers as 3 months before conception to 2 weeks afterward. The periconceptional period for recommended folic acid use is defined as 4 weeks before conception to 8 weeks afterward.

Both case and control parents filled in a questionnaire at home on demographics and on their periconceptional and first-trimester smoking, alcohol consumption, medication use and maternal folic acid supplementation. The mothers were asked to fill in questionnaires for the period covering 3 months before conception to 3 months afterward, while the fathers reported on the period of 3 months before conception to 2 weeks afterward. The questions on medication use asked about the type of medication, dosage, and frequency of intake, and the period in which medication was taken.

Maternal folic acid supplementation was defined as any taken during the periconceptional period, with a daily intake of at least 400 µg of folic acid, either in a multivitamin supplement or as a single tablet of folic acid from 4 weeks before conception to 8 weeks afterward, as recommended by the Dutch government for all women who want to become pregnant (Health Council and Food and Nutrition Council 1993). There were also questions on the mother's periconceptional use of multivitamins with or without folic acid.

Both parents were also asked about their family history and if they reported any family member with an OFC, the family history was defined as positive.

We selected case and control children and their parents for whom DNA was available and the *MSX1* CA repeats could be successfully analyzed. This resulted in 181 case mothers and 132 control mothers, 155 case fathers and 121 control fathers, and 176 cases and 146 control children. Due to the poor quality of the DNA isolated from a number of buccal swabs, CA repeat data were only available for 107 complete case triads and for 66 complete control triads to be included in our TDT analysis.

Determination of serum and red blood cell folate

A venous blood sample was taken from the mothers to measure their concentrations of serum and red blood cell (RBC) folate. These were measured using a microbiologic assay. Sample collection and laboratory determination were described previously (van Rooij et al. 2003).

Genotyping

The analyses were based on an intronic polymorphic CA repeat in the *MSX1* gene. The CA repeat alleles were determined by polymerase chain reaction (PCR) and fragment analysis. Primers and conditions for PCR were as described previously, with minor modifications (Hwang et al. 1998). After amplifying the DNA, PCR products were run on an ABI 3100 sequencer (Applied Biosystems) and analyzed using genescan and genotyper software (Applied Biosystems). Sequencing analysis was performed on representative samples to determine the exact repeat numbers for different alleles (data not shown). Four different alleles could be identified and were called as in previous studies: allele 1, 12 CA repeats; allele 2, 11 CA repeats; allele 3, 10 CA repeats; allele 4, 9 CA repeats (Jugessur et al. 2003).

Statistical analysis

Sample characteristics were compared between cases and controls using *t* test or Chi-square tests. The frequencies of the four *MSX1* CA repeat alleles were compared between cases and controls, and odds ratios (OR) were derived with 95% confidence intervals (CI), for which allele 1 served as the reference. To test for genetic association in nuclear families, we used the software package UNPHASEDv2.4, which implements a likelihood-based approach allowing for missing parental data (Dudbridge 2008).

The genotypes for the *MSX1* CA repeat were allotted to three genotype categories defined in previous studies: CA4 homozygotes (4/4), CA4 heterozygotes (4/x), and CA4 non-carriers (x/x) (Beaty et al. 2002; Fallin et al. 2003; Jugessur et al. 2003). Genotypic odds ratios with 95% CI were derived. We investigated gene–environment interactions by further stratifying environment factors such as parental smoking, alcohol consumption, medication use, and maternal folic acid supplementation.

Because CA4 is the most common allele, the groups of non-exposed CA4 non-carriers were too small to serve as a reference. We therefore combined CA4 heterozygotes and CA4 non-carriers (4/x, x/x) into one category for all the stratified and non-stratified

analyses. The limited sample size meant stratified analyses of the different OFC phenotypes were not feasible.

Pooling different types of clefting is consistent with the finding that *MSX1* is involved in both CL/P and CPO (van den Boogaard et al. 2000). Furthermore, a recent study identified occult lip defects with high-resolution ultrasound of the upper lip in a subset of CP cases, showing how difficult it is to classify orofacial clefting in different types (Weinberg et al. 2008).

The data for folic acid values were compared between the groups using Wilcoxon's rank sum test.

Results

Characteristics

The characteristics of the case and control groups are given in Table 5.1. Case children were almost twice as likely to be boys compared to controls (OR 1.8, 95% CI 1.1–2.8), consistent with the large number of CL/P cases and known male predominance (Jugessur et al. 2003). There were no significant differences in the characteristics of parents and children between the cases and controls, except that a positive family history was reported significantly more frequently by case parents compared to controls (both $P < 0.01$)

Table 5.1 Characteristics of the case and control triads

Table 5.1

	Cases	%	Missing	Controls	%	Missing	OR (95% CI)	*P* value
Child	*n* = 176			*n* = 146				
Age at the study moment in months, mean (SD)	15.2	4.2	2	14.7	6.7	6		0.43
Boys, *n* (%)	114	64.8		74	50.7		1.8 (1.1–2.8)	0.01
Boys in CL/P cases, *n* (%)	102	68.0		74	50.7		2.1 (1.3–3.4)	<0.01
Boys in CP cases, *n* (%)	12	46.2		74	50.7		0.8 (0.4–1.9)	0.65
Type of OFC								
CL, *n* (%)	58	33.0						
CP, *n* (%)	26	14.8						
CLP, *n* (%)	92	52.3						
Mother	*n* = 181			*n* = 132				0.54
Age, years, mean (SD)[a]	30.6	3.9	1	30.9	3.8	3		
Family history for OFC positive, *n* (%)	38	21.1	1	11	8.4	1		<0.01
Periconception, yes, *n* (%)								
Smoking[b]	52	28.9	1	27	20.6	1	1.6 (0.9–2.7)	0.10
Alcohol[c]	68	37.8	1	60	45.8	1	0.7 (0.5–1.1)	0.16
Medication[d]	68	37.8	1	26	19.9	1	2.5 (1.5–4.1)	<0.01
Any folic acid use[e]	113	62.8	1	94	71.8	1	0.7 (0.4–1.1)	0.10
Folic acid use/multivitamin use[f]	54	44.6	60	56	60.2	39	0.5 (0.3–0.9)	0.02
Father	*n* = 155			*n* = 121		1		
Age, years, mean (SD)	33.1	4.1	3	33.8	4.8			0.21
Family history for OFC positive, *n* (%)	33	21.9	4	10	8.5	3		<0.01
Periconception, yes, *n* (%)								
Smoking[b]	73	47.7	2	43	35.8	1	1.6 (1.0–2.7)	0.05
Alcohol[c]	137	89.5	2	105	87.5	1	1.2 (0.6–2.6)	0.60
Medication[d]	26	17.0	2	12	10.0	1	1.8 (0.9–3.8)	0.10

SD standard deviation, CL/P cleft lip with or without cleft palate, CL cleft lip, CP cleft palate, CLP cleft lip with cleft palate; OFC orofacial clefting;
OR (95% CI) = odds ratio, 95% confidence ; n = number for whom MSX1 CA repeat alleles were successfully analyzed; P value tested by Chi-square
a At delivery of the child
b Any smoking/cigarettes/cigar/pipe
c Any alcohol use
d Any medication other than oral contraceptives or iron
e Any use of supplements containing folic acid
f Daily use of supplements containing folic acid with a minimum of 400 µg folic acid from 4 weeks before conception to 8 weeks afterward, one case mother used 5 mg per day. Incidental users and women who started folic acid supplements later than 4 weeks before conception were excluded

In the periconceptional period, case mothers used significantly more medication ($P < 0.01$) and case fathers smoked more than controls ($P = 0.05$). However, maternal medications were rather diverse and included analgesics, decongestive nose-sprays, antibiotics, antimycotics, antihistamic drugs, ovulation-inducing drugs, antidepressive drugs and thyroid drugs. No anti-epileptic drugs, nor vitamin A or its congeners, were reported.

There was no significant difference in the use of folic acid between case and control mothers. In total, 62.8% of case mothers and 71.8% of control mothers took some folic acid in the periconceptional period. However, when maternal folic acid supplementation was defined as daily use from 4 weeks before conception to 8 weeks afterward, case mothers took significantly less folic acid supplementation. Of those who took supplements, all but one mother used 400 µg folic acid; this case mother took 5 mg folic acid daily during the recommended period without clear indication.

Serum and red blood cell folate levels

The median serum and red blood cell (RBC) folate concentrations were within the normal range (>4.8 and >340 nmol/L, respectively) (van Rooij et al. 2003) (Table 5.2). The median level of serum folate was lower in the case mothers than in control mothers, 13.2 and 15.2 nmol/L. The median level of serum folate was lower in mothers who smoked during the periconceptional period than in non-smoking mothers, 12.7 and 14.0 nmol/L. In mothers who smoked and who had the 4/4 genotype the median level of serum folate was lower, 11.3 nmol/L, than in non-smoking 4/4 mothers, 13.0 nmol/L. In addition, the median level of serum folate was lower in case mothers who smoked and who had the 4/4 genotype than in those smoking case mothers not carrying the 4/4 genotype (category 4/x, x/x), 11.3 and 14.5 nmol/L

Table 5.2 Plasma and red blood cell folate concentrations in relation to smoking and *MSX1* genotype

Table 5.2

	Case mothers	**Control mothers**	***P* value***
	n = 101	*n* = 80	
	Median (p5–p95)	Median (p5–95)	
Plasma folate (nmol/L)	13.2 (5.7–38.6)	15.2 (6.8–58.3)	0.40
	n = 103	*n* = 82	
Red blood cell folate (nmol/L)	654.5 (325.6–1226.5)	634.8 (348.3–1418.6)	0.61
	Smoking mothers	**Non-smoking mothers**	***P* value***
	n = 44	*n* = 136	
	Median (p5–p95)	Median (p5–p95)	
Plasma folate (nmol/L)	12.7 (5.2–38.6)	14.0 (6.6–55.9)	0.30
	n = 44	*n* = 139	
Red blood cell folate (nmol/L)	641.8 (266.8–1182.2)	637.8 (342.8–1401.9)	0.26
	Smoking 4/4 mothers	**Non-smoking 4/4 mothers**	***P* value***
	n = 12	*n* = 46	
	Median (p5–p95)	Median (p5–p95)	
Plasma folate (nmol/L)	11.3 (5.1–26.5)	13.0 (5.9–48.2)	0.19
	n = 12	*n* = 46	
Red blood cell folate (nmol/L)	527.6 (165.7–1024.9)	594.0 (363.5–1446.1)	0.16
	Smoking 4/4 case mothers	**Smoking 4/x, x/x case mothers**	***P* value***
	n = 10	*n* = 20	
	Median (p5–p95)	Median (p5–p95)	
Plasma folate (nmol/L)	11.3 (5.1–26.5)	14.5 (5.1–38.7)	0.19
	n = 10	*n* = 20	
Red blood cell folate (nmol/L)	554.9 (165.7–1024.9)	681.9 (246.7–1204.4)	0.59

* Wilcoxon's rank sum test

The median RBC folate concentrations also revealed no significant differences. The median RBC folate level in case mothers was 654.5 nmol/L, while it was 634.8 nmol/L in control mothers. The median RBC folate in mothers who smoked during the periconceptional period was 641.8 nmol/L, and in non-smoking mothers it was 637.8 nmol/L. The RBC folate concentration was lower in mothers who smoked and had the 4/4 genotype than in non-smoking 4/4 mothers, 527.6 and 594.0 nmol/L, respectively. Furthermore, the median level of RBC folate was lower in case mothers who smoked and had the 4/4 genotype than in case mothers who smoked but did not have the 4/4 genotype (category 4/x, x/x), 554.9 and 681.9 nmol/L, respectively.

CA repeat alleles and *MSX1* genotypes

As in previous studies, four alleles were identified (Jugessur et al. 2003). The distributions of the CA repeat alleles and genotypes were not significantly different among case and control children or among their parents. Allele 4 (nine repeats of the CA marker in the *MSX1* allele) was the most common allele in case children (58.0%), mothers (57.7%), and fathers (53.9%), and in control children (56.1%), mothers (53.4%), and fathers (55.8%), regardless of OFC status (Fig. 5.1; Table 5.3).

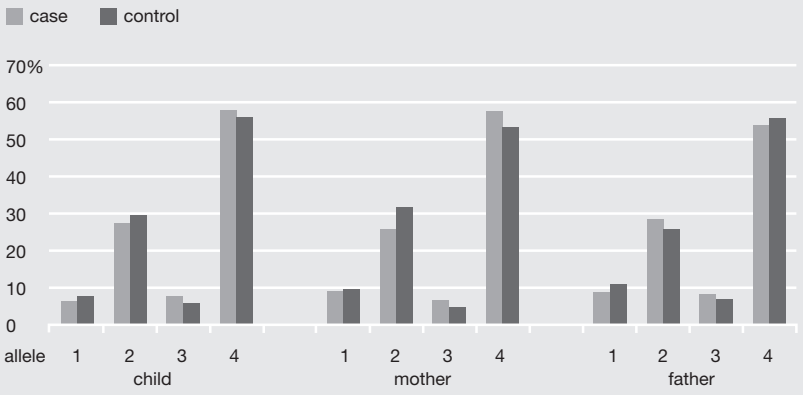


Figure 5.1

Figure 5.1 Allele frequencies (%) in case and control child, mother, and father.

Table 5.3 Case-control genotype distribution of the *MSX1* CA repeat in children, mothers and fathers in association with risk of orofacial clefting

Table 5.3

Genotype	No. of cases	%	No. of controls	%	OR (95% CI)
Child	*n* = 176		*n* = 146		
4/4	56	31.8	45	30.8	1.1 (0.7–1.7)
4/x, x/x[a]	120	68.2	101	69.2	1.0 (Reference)
Mother	*n* = 181		*n* = 132		
4/4	61	33.7	33	25.0	1.5 (0.9–2.5)
4/x, x/x[a]	120	66.3	99	75.0	1.0 (Reference)
Father	*n* = 155		*n* = 121		
4/4	44	28.4	37	30.6	0.9 (0.5–1.5)
4/x, x/x[a]	111	71.6	84	69.4	1.0 (Reference)

[a] Combined genotype category, x = allele 1, 2, 3

For the TDT analysis, we analyzed 107 informative case triads. Case fathers transmitted allele 1 on three occasions but did not transmit allele 1 on 14 occasions (P = 0.02).

Genotype 1/4 was seen less frequently in cases than in controls (OR 0.47, 95% CI 0.2–1.1). However, the frequency of allele 1 was low and the numbers were too small for further analysis.

Interactions between lifestyle factors and MSX1 genotypes

Gene–environment interaction analysis for parental and child genotype and parental smoking, alcohol use, medication use and maternal folic acid supplement use was performed and is shown in Tables 5.4 and 5.5.

Table 5.4 Interaction between parental smoking during periconceptional period and the *MSX1* CA repeat genotype on the risk of orofacial clefting

Genotype	Smoking	No. of cases	%	No. of controls	%	OR (95% CI)
Child	*Mother*	*n* = 175		*n* = 145		
4/4	Yes	23	13.1	7	4.8	2.7 (1.1–6.6)
4/x, x/x[a]	Yes	30	17.1	28	19.3	0.9 (0.5–1.6)
4/4	No	33	18.9	38	26.2	0.7 (0.4–1.2)
4/x, x/x[a]	No	89	50.9	72	49.7	1.0 (Reference)
Child	*Father*	*n* = 172		*n* = 143		
4/4	Yes	28	16.3	12	8.4	2.2 (1.0–4.7)
4/x, x/x[a]	Yes	55	32.0	39	27.3	1.3 (0.8–2.3)
4/4	No	26	15.1	33	23.1	0.7 (0.4–1.4)
4/x, x/x[a]	No	63	36.6	59	41.3	1.0 (Reference)
Child	*Father* [b]	*n* = 119		*n* = 108		
4/4	Yes	12	10.1	9	8.3	1.2 (0.5–3.2)
4/x, x/x[a]	Yes	32	26.9	18	16.7	1.7 (0.8–3.3)
4/4	No	19	16.0	29	26.9	0.6 (0.3–1.2)
4/x, x/x[a]	No	56	47.0	52	48.1	1.0 (Reference)
Child	*Mother + father* [c]	*n* = 114		*n* = 105		
4/4	Yes	16	14.0	3	2.9	4.9 (1.4–18.0)
4/x, x/x[a]	Yes	23	20.2	21	20.0	1.0 (0.5–2.1)
4/4	No	19	16.7	29	27.6	0.6 (0.3–1.2)
4/x, x/x[a]	No	56	49.1	52	49.5	1.0 (Reference)
Mother		*n* = 180		*n* = 131		
4/4	Yes	18	10.0	5	3.8	3.2 (1.1–9.0)
4/x, x/x[a]	Yes	34	18.9	22	16.8	1.4 (0.7–2.5)
4/4	No	42	23.3	28	21.4	1.3 (0.8–2.3)
4/x, x/x[a]	No	86	47.8	76	58.0	1.0 (Reference)
Father		*n* = 153		*n* = 120		
4/4	Yes	22	14.4	12	10.0	1.6 (0.7–3.6)
4/x, x/x[a]	Yes	51	33.3	31	25.8	1.5 (0.8–2.6)
4/4	No	21	13.7	25	20.8	0.7 (0.4–1.5)
4/x, x/x[a]	No	59	38.6	52	43.3	1.0 (Reference)

OR (95% CI) = odds ratios 95% confidence interval

[a] Combined genotype category x = allele 1, 2, 3

[b] Independent effect of smoking by the father, children of two smoking parents were excluded

[c] Mother + father; *yes* both parents smoking, *no* both parents not smoking

Table 5.5 Folic acid use, *MSX1* CA repeat genotype and risk of orofacial clefting

Table 5.5

a. Interaction between maternal folic acid use in the recommended periconceptional period and *MSX1* CA repeat genotype on the risk of orofacial clefting

Genotype	Folic acid [b]	No. of cases	%	No. of controls	%	OR (95% CI)
Child	*Mother*	*n* = 117		*n* = 106		
4/4	No	23	19.7	13	12.3	2.0 (0.9–4.5)
4/x, x/x[a]	No	44	37.6	35	33.0	1.4 (0.7–2.7)
4/4	Yes	15	12.8	19	17.9	0.9 (0.4–2.0)
4/x, x/x[a]	Yes	35	29.9	39	36.8	1.0 (Reference)
Mother		*n* = 121		*n* = 93		
4/4	No	22	18.2	9	9.7	2.8 (1.1–6.7)
4/x, x/x[a]	No	45	37.2	28	30.1	1.8 (0.96–3.5)
4/4	Yes	16	13.2	13	14.0	1.4 (0.6–3.3)
4/x, x/x[a]	Yes	38	31.4	43	46.2	1.0 (Reference)

b. Interaction between some maternal folic acid use during pregnancy and *MSX1* CA repeat genotype on the risk of orofacial clefting

Genotype	Folic acid [c]	No. of cases	%	No. of controls	%	OR (95% CI)
Child	*Mother*	*n* = 175		*n* = 145		
4/4	No	23	13.1	13	9.0	1.5 (0.7–3.3)
4/x, x/x[a]	No	44	25.1	35	24.1	1.1 (0.6–1.9)
4/4	Yes	33	18.9	32	22.1	0.9 (0.5–1.6)
4/x, x/x[a]	Yes	75	42.9	65	44.8	1.0 (Reference)
Mother		*n* = 180		*n* = 131		
4/4	No	22	12.2	9	6.9	2.3 (0.98–5.3)
4/x, x/x[a]	No	45	25.0	28	21.4	1.5 (0.9–2.7)
4/4	Yes	38	21.1	24	18.3	1.5 (0.8–2.7)
4/x, x/x[a]	Yes	75	41.7	70	53.4	1.0 (Reference)

OR (95% CI) = odds ratios 95% confidence interval

[a] Combined genotype category x = allele 1, 2, 3; [b] Daily use of supplements containing folic acid (400–500 µg) from 4 weeks before conception of the child until 8 weeks after; incidental users and women who started folic acid supplements later than 4 weeks before conception were excluded; [c] Any folic acid use during pregnancy, also incidental users and women who started folic acid supplements later than 4 weeks before conception were included

Maternal smoking influenced the risk for OFC if either the child or mother was homozygous for allele 4 (OR 2.7, 95% CI 1.1–6.6 and OR 3.2, 95% CI 1.1–9.0, respectively) (Table 5.4). If both parents smoked, the odds ratio was increased to 4.9 (95% CI 1.4–18.0) (Table 5.4). The possible effects of paternal smoking are presented in Table 5.4. Children homozygous for allele 4 of fathers who smoked showed a more than twofold increased OFC risk (OR 2.2, 95% CI 1.0–4.7). When we excluded the children with two smoking parents from this analysis the odds ratio was 1.2 (95% CI 0.5–3.2).

An association was found between the mother not taking folic acid supplements and increased OFC risk (Table 5.5 a, b). Not taking folic acid daily in the recommended period increased OFC risk for children homozygous for allele 4 (4/4) (OR 2.0, 95% CI 0.9–4.5) as well as for children not carrying the 4/4 genotype (category 4/x, x/x) (OR 1.4, 95% CI 0.7–2.7) (Table 5a). In addition, not taking folic acid supplements daily during the recommended period increased the risk of having a child with OFC in both mothers homozygous for allele 4 (4/4) (OR 2.8, 95% CI 1.1–6.7) and in mothers without the 4/4 genotype (category 4/x, x/x) (OR 1.8, 95% CI 0.96–3.5) (Table 5.5 a). Only a relatively small number of mothers (54 case mothers and 56 control mothers) took the advised 400 µg folic acid daily in the recommended period. The analyses were therefore repeated for mothers taking any folic acid supplements at all (Table 5.5 b).

During pregnancy, case mothers more often took medication (OR 2.5, 95% CI 1.5–4.1) (Table 5.1). The OFC risk was higher in mothers or their children homozygous for allele 4 if the mother used medication (OR 8.3, 95% CI 2.4–28.7 and OR 1.9, 95% CI 0.9–4.0, respectively), albeit not significantly in the children. If fathers used medication and they or their children were homozygous for allele 4, the OFC risk was also increased, albeit not significantly (OR 3.3, 95% CI 0.9–12.0 and OR 1.2, 95% CI 0.5–3.1, respectively). We found no significant interactions between the 4/4 genotype in mothers, fathers, or children, parental alcohol use and OFC risk (data not shown).

Discussion

This study provides further evidence for an association between the homeobox gene *MSX1* and maternal smoking during the first trimester of pregnancy and offspring with nonsyndromic OFC. In addition, the daily use of folic acid supplement from 4 weeks before conception to 8 weeks afterward reduces OFC risk independent of *MSX1*

genotype. The analyses are based on an intronic polymorphic CA repeat in the *MSX1* gene, which identifies four alleles. Consistent with previous observations, allele 4 (nine repeats of the CA marker in the *MSX1* allele) was the most common allele (Jugessur et al. 2003). Our analysis suggests that allele 4 homozygous (4/4) children exposed to periconceptional smoking by both parents, but particularly by the mother, are more susceptible to OFC. The maternal 4/4 genotype and smoking showed a threefold higher OFC risk.

Several hypotheses might explain the mechanisms by which interaction between smoking and *MSX1* influences OFC risk. Studies in mice suggest that maternal cigarette smoke alters the expression of a large number of genes in the fetus. Especially genes involved in response to oxidative stress, DNA and protein repair, signal transduction and cell cycle regulation (CDK4 and CDK6 inhibitors) were upregulated (Izzotti et al. 2003). Interestingly, *Msx1* permits cell cycle progression and proliferation of tissue by inhibiting the expression of CDK inhibitors, resulting in E2F mediated expression of cell cycle genes (Han et al. 2003).

Treatment of mouse hepatoma cells with a component of tobacco smoke [2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)] resulted in the co-repression of E2F-mediated expression of genes necessary for cell cycle progression (Huang and Elferink 2005).

Another interesting hypothesis is that *AhR* suppresses *Tgfb3* expression and TGFB3 has an indirect effect on IRF6. Various studies have shown that both genes play a significant role in the development of the palate and lip (Murray and Schutte 2004). The finding of two IRF binding sites in the promotor and intron of *Msx1* indicates both genes might be part of a common pathway (Kondo et al. 2002). This would suggest that there is a relation between *Ahr* and *Msx1*, and thus indirectly between smoking and *MSX1*. The effect of the interaction between smoking and *Msx1* might also be a result of gene–gene interaction with genes involved in detoxification pathways. Results of a preliminary analysis suggested gene–gene interaction between *MSX1* with *CYP1B1*, *GSTM1*, *HIF1A*, and *SULT1A1* (Shi et al. 2007).

Another aspect of smoking by the mother is its possible influence on folate levels. It has been suggested that exposure to tobacco smoke decreases serum folate level and that these lower levels are not completely due to differences in folate intake from diet or

supplements (Mannino et al. 2003; Honein et al. 2007). In our study the current level of serum folate was also lower in the mothers who smoked during the periconceptional period than in the non-smoking mothers. This was in contrast to the RBC folate levels. The smoking mothers with the 4/4 genotype had a lower serum and RBC folate level than non-smoking 4/4 mothers. In addition, the level of serum and RBC folate was lower in the case mothers who smoked and had the 4/4 genotype than in the case mothers who smoked without this genotype. However, the numbers are too small to draw any firm conclusions.

Interestingly, knockout studies in mice demonstrated inactivation of the folate-binding protein Folbp1, decreased folate levels and clefting (Tang and Finnell 2003). The *Shh* expression was decreased in the facial primordial in the Folbp1 mutants, accompanied by an increase of *Bmp4* expression. It is striking that *Msx1* plays a significant role in the expression of *Bmp4*, and that *Msx1*, *Bmp4*, *Shh* and Bmp2 constitute a pathway essential in the palatogenesis in mice (Zhang et al. 2002). *Pax3* was also found to be upregulated in the nasal processes of the *Folbp1* mutant (Tang and Finnell 2003). In vivo studies showed that complex formation between MSX1 and PAX3 may prevent premature activation of myogenic genes (*MyoD*) in muscle precursor cells, which is important for the maintenance of their proliferative capacity and outgrowth (Bendall and Abate-Shen 2000). We speculate that low levels of folate might influence the function of specific *MSX1* variants, thereby affecting the *MSX1*, *BMP4*, *SHH* and *BMP2* pathway in the embryo. The downregulation of *Shh* affects the expression of *Pax3*, resulting in abnormal outgrowth of tissue. Further studies are needed to evaluate these possible mechanisms and the specific role of *MSX1* in these models in relation to OFC.

An interesting aspect of folic acid is that it functions as a one-carbon donor for DNA methylation, which is important in regulating gene expression. A recent study indicated expression of Msx1 in embryonic stem cells was regulated by a unique histone modification, which consisted of activating and repressive methylation within their promotor loci (Gan et al. 2007). We cannot rule out that low folate affects this mechanism as well, and it might explain potential associations between smoking, folic acid, *MSX1* and OFC risk.

In this study, only a relatively small number of mothers took the daily 400 µg folic acid in the recommended period. Association between supplemental folic acid use, *MSX1* and OFC risk was observed. Homozygosity for allele 4 in the child or mother combined with

the mother not taking folic acid supplements tended to increase OFC risk. However, any interaction between periconceptional folic acid intake and a specific *MSX1* genotype on OFC risk will have a relatively small effect.

Our case fathers smoked more often than control fathers, and paternal smoking may also contribute to OFC risk. Paternal smoking increases maternal exposure to tobacco smoke by passive smoking, thus indirectly influencing the environment of the fetus. Homozygosity for allele 4 in children and paternal smoking showed a twofold higher OFC risk. However, to exclude any possible effect from the mother smoking, we performed an additional analysis which excluded children with two smoking parents. The suggested interaction between paternal smoking, homozygosity for allele 4 in the child and OFC risk could not be confirmed. But again, our sample size was very small.

In all our analyses we used the intronic CA repeat marker. Recent studies (Knight 2004; Spielman et al. 2007) have revealed that polymorphic variants can also be responsible for individual differences in expression level, and specific genetic variation among populations contributes appreciably to differences in gene expression phenotypes. These variations of gene expression and specific gene expression phenotypes could account for a large proportion of susceptibility to complex genetic disorders (Spielman et al. 2007). Given this observation, the CA repeat may also play a role in the etiology of clefting. The *Msx1* gene encodes an antisense RNA, which regulates gene expression, and the antisense strand, in the 3'UTR to the middle of the intron, includes the CA repeat (Blin-Wakkach et al. 2001). We hypothesize that differences in the CA repeat numbers of the opposite alleles may alter the binding between the sense and antisense RNA molecules of both alleles and could thereby affect regulation of gene expression. This could be an explanation for the possibly protective effect of the 1/4 genotype and our finding that this genotype was more prevalent in controls than in cases. The difference in the repeat numbers of the opposite alleles was most profound in the 1/4 genotype, which may influence regulation of gene expression by antisense RNA. Further studies are needed to evaluate this possible mechanism.

We performed our study using a case-control study design, in which recall bias of periconceptional exposure must be considered. To minimize this issue, we used a standardized study moment, when the child was around 15 months old for both the case and control groups. This moment is in the same season as the periconceptional period and just after the first birthday, which may facilitate parents' recall. However, several

studies have indicated that the role of recall bias in case-control studies focusing on congenital malformations can be considered fairly small (Infante-Rivard and Jacques 2000) and our data on lifestyle exposures of the reproductive age group also correspond with those reported in the Dutch population (Statistics Netherlands 2003).

In conclusion, we demonstrate a complex role for the *MSX1* gene, maternal smoking, and folic acid intake in the etiology of OFC and show some interesting gene–environment interactions that influence the risk of OFC. Further studies are needed to resolve the details of the pathogenic mechanisms and to determine the genetic and environmental risk factors playing a decisive role in the occurrence of OFC. Ultimately, this work may be helpful in providing better tailored advice to individuals in preconceptional counseling.

Acknowledgements

We thank the nine Dutch cleft lip and palate teams, the organization for Dutch patients with clefts and their parents (BOSK), and their coordinators (Mr B. van Beek, BOSK; Dr W. Brussel, Rijnstate Hospital Arnhem; Prof. B. Prahl-Andersen, Free University Hospital Amsterdam and Erasmus MC University Medical Center Rotterdam; Prof. S. M. Goorhuis-Brouwer, University Medical Center Groningen; Dr. J. J. van der Biezen, Medical Center Leeuwarden; Prof A. M. Kuijpers-Jagtman, Radboud University Nijmegen Medical Center; Dr. J. G. Daggers, St. Elisabeth Hospital Tilburg; Dr. A. B. Mink van der Molen, University Medical Center Utrecht; and Dr. P. Houpt, Sophia Hospital Zwolle). We thank the nurseries and public health centers for their help in recruiting control mothers. We are very grateful to Mrs. M. van der Doelen, chief nursing officer, and Dr. C. van Oostrom, head of the outpatient pediatric clinic, Radboud University Nijmegen Medical Center, for their assistance; Dr. C. Vermeij-Keers for the opportunity to use the registration system of the Dutch Association for Cleft Palate and Craniofacial Anomalies (BOSK). We thank P. van Zon and G-J. de Jong for laboratory assistance and J. Senior for reading the manuscript.

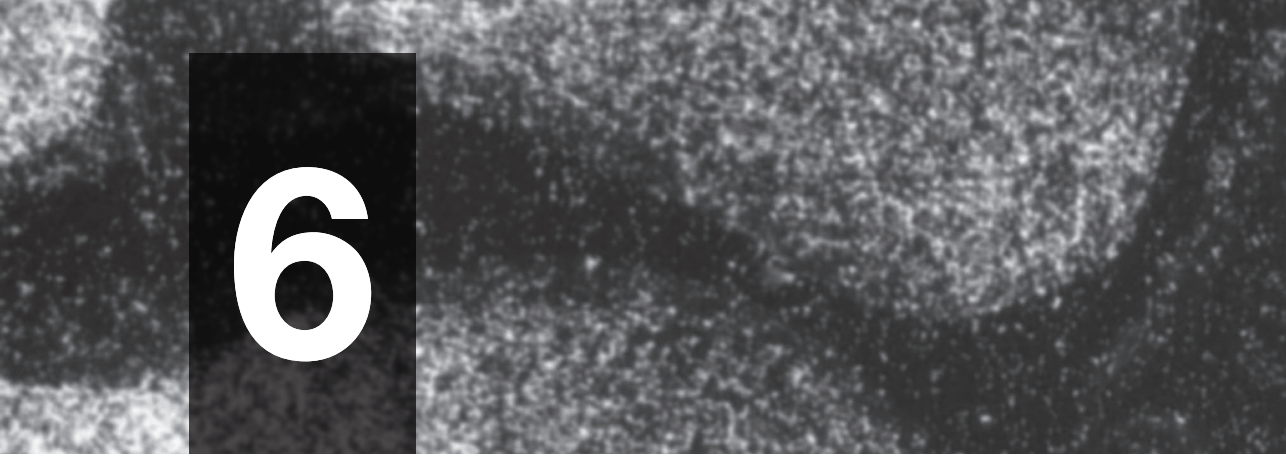
References

1. Beaty TH, Wang H, Hetmanski JB, Fan YT, Zeiger JS, Liang KY, Chiu YF, Vanderkolk CA, Seifert KC, Wulfsberg EA, Raymond G, Panny SR, McIntosh I. A case-control study of nonsyndromic oral clefts in Maryland. Ann Epidemiol 2001;11:434–442
2. Beaty TH, Hetmanski JB, Zeiger JS, Fan YT, Liang KY, VanderKolk CA, McIntosh I. Testing candidate genes for non-syndromic oral clefts using a case-parent trio design. Genet Epidemiol 2002;22:1–11
3. Bendall AJ, Abate-Shen C. Roles for Msx and Dlx homeoproteins in vertebrate development. Gene 2000;247:17–31
4. Blanco R, Suazo J, Santos JL, Paredes M, Sung H, Carreño H, Jara L. Association between 10 microsatellite markers and nonsyndromic cleft lip palate in the Chilean population. Cleft Palate Craniofac J 2004;41:163–167
5. Blin-Wakkach C, Lezot F, Ghoul-Mazgar S, Hotton D, Monteiro S, Teillaud C, Pibouin L, Orestes-Cardoso S, Papagerakis P, Macdougall M, Robert B, Berdal A. Endogenous Msx1 antisense transcript: in vivo and in vitro evidences, structure, and potential involvement in skeleton development in mammals. Proc Natl Acad Sci USA 2001;98:7336–7341
6. Davidson D. The function and evolution of Msx genes: pointers and paradoxes. Trends Genet 1995;11:405–411
7. Dudbridge F. Likelihood-based association analysis for nuclear families and unrelated subjects with missing genotype data. Hum Hered 2008;66:87–98
8. Etheredge AJ, Christensen K, Del Junco D, Murray JC, Mitchell LE. Evaluation of two methods for assessing gene-environment interactions using data from the Danish case-control study of facial clefts. Birth Defects Res A Clin Mol Teratol 2005;73:541–546
9. Fallin MD, Hetmanski JB, Park J, Scott AF, Ingersoll R, Fuernkranz HA, McIntosh I, Beaty TH. Family based analysis of MSX1 haplotypes for association with oral clefts. Genet Epidemiol 2003;25:168–175
10. Gan Q, Yoshida T, McDonald OG, Owens GK. Concise review: epigenetic mechanisms contribute to pluripotency and cell lineage determination of embryonic stem cells. Stem Cells 2007;25:2–9
11. Gritli-Linde A. Molecular control of secondary palate development. Dev Biol 2007;301:309–326
12. Han J, Ito Y, Yeo JY, Sucov HM, Maas R, Chai Y. Cranial neural crest-derived mesenchymal proliferation is regulated by Msx1-mediated p19(INK4d) expression during odontogenesis. Dev Biol 2003;261:183–196
13. Health Council & Food and Nutrition Council (1993) Follow-up report on the relationship between folic acid intake and neural tube defects. Health Council & Food and Nutrition Council, The Hague
14. Honein MA, Rasmussen SA, Reefhuis J, Romitti PA, Lammer EJ, Sun L, Correa A. Maternal smoking and environmental tobacco smoke exposure and the risk of orofacial clefts. Epidemiology 2007;18:226–233
15. Huang G, Elferink CJ. Multiple mechanisms are involved in Ah receptor-mediated cell cycle arrest. Mol Pharmacol 2005;67:88–96
16. Hwang SJ, Beaty TH, McIntosh I, Hefferon T, Panny SR. Association between homeobox-containing gene MSX1 and the occurrence of limb deficiency. Am J Med Genet 1998;75:419–423

17. Infante-Rivard C, Jacques L. Empirical study of parental recall bias. Am J Epidemiology 2000;152:480–486
18. Izzotti A, Balansky RM, Cartiglia C, Camoirano A, Longobardi M, De Flora S. Genomic and transcriptional alterations in mouse fetus liver after transplacental exposure to cigarette smoke. FASEB J 2003;17:1127–1129
19. Jezewski PA, Vieira AR, Nishimura C, Ludwig B, Johnson M, O'Brien SE, Daack-Hirsch S, Schultz RE, Weber A, Nepomucena B, Romitti PA, Christensen K, Orioli IM, Castilla EE, Machida J, Natsume N, Murray JC. Complete sequencing shows a role for MSX1 in non-syndromic cleft lip and palate. J Med Genet 2003;40:399–407
20. Jugessur A, Murray JC. Orofacial clefting: recent insights into a complex trait. Curr Opin Genet Dev 2005;15:270–280
21. Jugessur A, Lie RT, Wilcox AJ, Murray JC, Taylor JA, Saugstad OD, Vindenes HA, Abyholm F. Variants of developmental genes (TGFA, TGFB3, and MSX1) and their associations with orofacial clefts: a case-parent triad analysis. Genet Epidemiol 2003;24:230–239
22. Knight JC. Allele-specific gene expression uncovered. Trends Genet 2004;20:113–116
23. Koillinen H, Ollikainen V, Rautio J, Hukki J, Kere J. Linkage and linkage disequilibrium searched for between non-syndromic cleft palate and four candidate loci. J Med Genet 2003;40:464–468
24. Kondo S, Schutte BC, Richardson RJ, Bjork BC, Knight AS, Watanabe Y, Howard E, de Lima RL, Daack-Hirsch S, Sander A, McDonald-McGinn DM, Zackai EH, Lammer EJ, Aylsworth AS, Ardinger HH, Lidral AC, Pober BR, Moreno L, Arcos-Burgos M, Valencia C, Houdayer C, Bahuau M, Moretti-Ferreira D, Richieri-Costa A, Dixon MJ, Murray JC. Mutations in IRF6 cause Van der Woude and popliteal pterygium syndromes. Nat Genet 2002;32:285–289
25. Krapels IP, Vermeij-Keers C, Müller M, de Klein A, Steegers-Theunissen RP. Nutrition and genes in the development of orofacial clefting. Nutr Rev 2006a;64:280–288
26. Krapels IP, Zielhuis GA, Vroom F, den Berg LT, Kuijpers-Jagtman AM, van der Molen AB, Steegers-Theunissen RP, Eurocran Gene-Environment Interaction Group. Periconceptional health and lifestyle factors of both parents affect the risk of live-born children with orofacial clefts. Birth Defects Res A Clin Mol Teratol 2006b;76:613–620
27. Lidral AC, Romitti PA, Basart AM, Doetschman T, Leysens NJ, Daack-Hirsch S, Semina EV, Johnson LR, Machida J, Burds A, Parnell TJ, Rubenstein JL, Murray JC. Association of MSX1 and TGFB3 with nonsyndromic clefting in humans. Am J Hum Genet 1998;63:557–568
28. Luijsterberg AMJ, Vermeij-Keers C (1999) Recording form and manual for congenital malformations of the head/neck area. ISBN 90-76580-02-2
29. Mannino DM, Mulinare J, Ford ES, Schwartz J. Tobacco smoke exposure and decreased serum and red blood cell folate levels: data from the Third National Health and Nutrition Examination Survey. Nicotine Tob Res 2003;5:357–362
30. Mitchell LE, Murray JC, O'Brien S, Christensen K. Evaluation of two putative susceptibility loci for oral clefts in the Danish population. Am J Epidemiol 2001;153:1007–1015
31. Modesto A, Moreno LM, Krahn K, King S, Lidral AC. MSX1 and orofacial clefting with and without tooth agenesis. J Dent Res 2006;85:542–546
32. Moreno LM, Arcos-Burgos M, Marazita ML, Krahn K, Maher BS, Cooper ME, Valencia-Ramirez CR, Lidral AC. Genetic analysis of candidate loci in non-syndromic cleft lip families from Antioquia–Colombia and Ohio. Am J Med Genet A 2004;125:135–144

33. Murray JC, Schutte BC. Cleft palate: players, pathways, and pursuits. J Clin Invest 2004;113:1676–1678
34. Park J, Park BY, Kim HS, Lee JE, Suh I, Nam CM, Kang DR, Kim S, Yun JE, Go EN, Jee SH, Beaty TH. MSX1 polymorphism associated with risk of oral cleft in Korea: evidence from case-parent trio and case-control studies. Yonsei Med J 2007;48:101–108
35. van Rooij IA, Groenen PM, van Drongelen M, Te Morsche RH, Peters WH, Steegers-Theunissen RP. Orofacial clefts and spina bifida: N-acetyltransferase phenotype, maternal smoking, and medication use. Teratology 2002;66:260–266
36. van Rooij IA, Swinkels DW, Blom HJ, Merkus HM, Steegers-Theunissen RP. Vitamin and homocysteine status of mothers and infants and the risk of nonsyndromic orofacial clefts. Am J Obstet Gynecol 2003;189:1155–1160
37. Satokata I, Maas R. Msx1 deficient mice exhibit cleft palate and abnormalities of craniofacial and tooth development. Nat Genet 1994;6:348–356
38. Schliekelman P, Slatkin M. Multiplex relative risk and estimation of the number of loci underlying an inherited disease. Am J Hum Genet 2002;71:1369–1385
39. Schutte CC, Murray JC. The many faces and factors of orofacial clefts. Hum Mol Genet Hum Mol Genet 1999;8:1853–1859
40. Shi M, Christensen K, Weinberg CR, Romitti P, Bathum L, Lozada A, Morris RW, Lovett M, Murray JC. Orofacial cleft risk is increased with maternal smoking and specific detoxification-gene variants. Am J Hum Genet 2007;80:76–90
41. Spielman RS, Bastone LA, Burdick JT, Morley M, Ewens WJ, Cheung VG. Common genetic variants account for differences in gene expression among ethnic groups. Nat Genet 2007;39:226–231
42. Tang LS, Finnell RH. Neural and orofacial defects in Folbp1 knockout mice. Birth Defects Res A Clin Mol Teratol 2003;67:209–218
43. Tongkobpetch S, Siriwan P, Shotelersuk V. MSX1 mutations contribute to nonsyndromic cleft lip in a Thai population. J Hum Genet 2006;51:671–676
44. Van den Boogaard MJ, Dorland M, Beemer FA, Ploos van Amstel HK. Msx1 mutation is associated with orofacial clefting and tooth agenesis in humans. Nat Genet 2000;24:342–343
45. Vieira AR, Orioli IM, Castilla EE, Cooper ME, Marazita ML, Murray JC. MSX1 and TGFB3 contribute to clefting in South America. J Dent Res 2003;82:289–292
46. Vieira AR, Avila JR, Daack-Hirsch S, Dragan E, Félix TM, Rahimov F, Harrington J, Schultz RR, Watanabe Y, Johnson M, Fang J, O'Brien SE, Orioli IM, Castilla EE, Fitzpatrick DR, Jiang R, Marazita ML, Murray JC (2005) Medical sequencing of candidate genes for nonsyndromic cleft lip and palate. PLoS Genet 1:e64
47. Weinberg SM, Brandon CA, McHenry TH, Neiswanger K, Deleyiannis FW, de Salamanca JE, Castilla EE, Czeizel AE, Vieira AR, Marazita ML. Rethinking isolated cleft palate: evidence of occult lip defects in a subset of cases. Am J Med Genet A 2008;146:1670–1675
48. Zhang Z, Song Y, Zhao X, Zhang X, Fermin C, Chen Y. Rescue of cleft palate in Msx1-deficient mice by transgenic Bmp4 reveals a network of BMP and Shh signaling in the regulation of mammalian palatogenesis. Development 2002;129:4135–4146

5



6

Chapter 6

WNT10A, rather than *MSX1* is a major cause of non-syndromic hypodontia

Mutations in WNT10A are present in more than half of isolated hypodontia cases.

van den Boogaard MJ, Créton M, Bronkhorst Y, van der Hout A, Hennekam E, Lindhout D, Cune M, Ploos van Amstel HK.

Published in: J Med Genet. 2012 May;49(5):327-31.

Abstract

Background

Dental agenesis is the most common, often heritable, developmental anomaly in humans. Mutations in *MSX1*, *PAX9*, *AXIN2* and the ectodermal dysplasia genes *EDA*, *EDAR* and *EDARADD* have been detected in familial severe tooth agenesis. However, until recently, in the majority of cases (~90%) the genetic factor could not be identified, implying that other genes must be involved. Recent insights into the role of *Wnt10A* in tooth development, and the finding of hypodontia in carriers of the autosomal recessive disorder, odontoonychodermal dysplasia, due to mutations in *WNT10A* (OMIM 257980; OODD), make *WNT10A* an interesting candidate gene for dental agenesis.

Methods

In a panel of 34 patients with isolated hypodontia, the candidate gene *WNT10A* and the genes *MSX1, PAX9*, *IRF6* and *AXIN2* have been sequenced. The probands all had isolated agenesis of between six and 28 teeth.

Results

WNT10A mutations were identified in 56% of the cases with non-syndromic hypodontia. *MSX1, PAX9* and *AXIN2* mutations were present in 3%, 9% and 3% of the cases, respectively.

Conclusion

The authors identified *WNT10A* as a major gene in the aetiology of isolated hypodontia. By including *WNT10A* in the DNA diagnostics of isolated tooth agenesis, the yield of molecular testing in this condition was significantly increased from 15% to 71%.

Introduction

Hypodontia, defined as the congenital absence of one or more permanent teeth, is the most common congenital anomaly in man. Excluding the third molar, in Europeans, 5.5% fail to develop one or more permanent teeth. [1,2] Congenital lack of six or more permanent teeth, again excluding the third molar (oligodontia), is observed in approximately 0.14% of the population and is highly heritable. [1–4] Congenital dental agenesis can occur as an isolated anomaly or as one of the features in a large variety of syndromes. [2,4–6] Hypodontia is also a common feature of ectodermal dysplasia (ED). [3,6]

ED involves the abnormal development of at least two of the ectodermal structures regarding teeth, hair, nails and sweat glands and is a clinically and genetically heterogeneous disorder. [7, 8] Genes associated with ED include *EDA*, *EDAR*, *EDARADD* and *WNT10A*. [7, 8]

Typically, homozygous mutations in *WNT10A* cause various EDs often corresponding to the odontoonychodermal dysplasia (OODD) and Schöpf-Schulz-Passarge syndrome, both combining classic ectodermal developmental anomalies (eg, hypohidrosis, hypotrichosis, nail dysplasia, lacrimal duct hypo/aplasia, hypo/oligodontia) with additional cutaneous features including facial telangiectases and palmoplantar keratoderma. Schöpf-Schulz-Passarge syndrome (SPSS) is distinguished by the presence of multiple eyelid cysts, histologically corresponding to apocrine hidrocystomas. OODD is apparently characterised by a smooth tongue (ie, hypoplasia of lingual papillae). [9–12] However, extreme variability of the associated clinical findings, including hypodontia and additional ectodermal features, may be observed in patients homozygous but also heterozygous for mutations in *WNT10A*. [10, 11]

Interestingly, Bohring et al. (2009) suggested that nearly 50% of heterozygotes for *WNT10A* mutations might display isolated ectodermal developmental defects such as missing teeth. [11] According to this original finding, more recently, Kantaputra and Sripathomsawat (2011) demonstrated segregation of a heterozygous *WNT10A* mutation in an American family with autosomal-dominant tooth agenesis without recognisable ectodermal features [13]

These observations prompted us to study the contribution of *WNT10A* mutations in comparison with mutations in other genes associated with hypodontia among isolated hypodontia patients who hypodontia status was ascertained in a tertiary dental clinic.

Methods

Participants

Individuals with apparent isolated dental agenesis of six or more permanent teeth visiting the Departments of Oral and Maxillofacial Surgery, Prosthodontics and Special Dental Care of the University Medical Center Utrecht (UMC Utrecht) and the St. Antonius Hospital, Nieuwegein, were referred to the Department of Medical Genetics of the UMC

Utrecht for syndrome diagnostics and genetic counselling. Tooth agenesis in the patients was assessed by clinical examination by the dentist and on panoramic radiographs (figure 6.1).

Figure 6.1

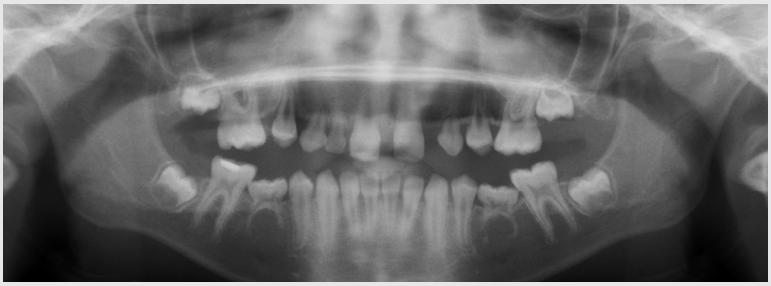


Figure 6.1 Panoramic radiograph of a non-syndromic *WNT10A* hypodontia patient (patient 4; table 6.1).

In total, 58 patients were referred. Thirteen of these patients were related. These patients were from six unrelated families and included three sib pairs (n=7), one parent-child pair, one pair of first cousins, and one uncle-niece pair. From each family, the oldest patient (n=6) referred was included in the patient cohort (n=51 patients), taking into account a potential age-related expression of additional features. In order to identify possible additional features of an ED or other syndromes, all patients were physically examined by a single clinical geneticist (MJvdB). In addition, patients were asked about possible symptoms of sweat glands, skin, hair and nails using a standardized form.

The patients were classified as displaying syndromic or non-syndromic hypodontia, based on the presence or absence of dysmorphic features or evident additional features (skin, hair, nails, sweat glands) suggestive of ED. Patients with one major additional ectodermal feature, more than two very mild additional ectodermal features, or with specific dysmorphic features, were classified as syndromic. Patients without additional symptoms, or with a very mild additional ectodermal feature of the skin and hair regarded as part of the normal spectrum in the general population, were classified as non-syndromic.

In total, 34 patients (14 men (41%) and 20 women (59%)) were classified as non-syndromic and included in this study. A mean of 14.6 (range: 6–28) teeth were missing.

The mean age of these patients was 19.7 years (range: 9–53). In 25 patients (73.5%), there was a positive family history (third degree or more closely related) for tooth agenesis. In 17 patients (10 men (59%) and seven women (41%)), the hypodontia was classified as suspect for ED or syndromic hypodontia due to their additional features (eg, sparse hair, nail abnormalities, cleft). The mean age of these patients was 20.5 years (range: 7–63 years).

Blood samples were obtained and DNA analyses of the genes *WNT10A*, *MSX1*, *PAX9*, *IRF6* and *AXIN2* were performed in both non-syndromic and syndromic cases. In the syndromic cases, additional DNA analysis was performed when a specific ED or syndrome was suspected.

When a mutation was detected, family members were asked to participate in this study. Data on tooth agenesis and possible additional ectodermal features were obtained from all participating family members. In total, 34 family members of *WNT10A* probands were available for DNA analysis.

Mutation analysis

High molecular weight genomic DNA was extracted from blood samples using standard procedures. PCR amplification of all exons and their splice site consensus sequences was performed with Amplitaq Gold 360 Master Mix (Applied Biosystems, Bedford, Massachusetts, USA). Sequencing of the MSX1 (NM_002448.3), PAX9 (NM_006194.2), IRF6 (NM_006147.2), AXIN2 (NM_004655.3) and WNT10A (NM_025216.2) genes was performed using the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). An ABI 3130, or 3730 sequencer (Applied Biosystems), was used for analysis. Mutation analysis was performed using the genetic analysis software Sequence Pilot V. 3.4.4 (JSI Medical Systems GmbH, Kippenheim, Germany), and mutation interpretation software Alamut (Interactive Biosoftware, Rouen, France) was used for further interpretation. Nomenclature is according HGVS guidelines.

Results

Genotyping

Mutation analysis of the exons and their flanking sequences of the genes *WNT10A*, *MSX1*, *PAX9*, *IRF6*, *AXIN2* in the 34 patients with non-syndromic hypodontia revealed mutations in 24 probands (71%). In 19 cases (56%), a mutation in *WNT10A* was identified: eight probands were homozygous, four probands were compound heterozygous and seven probands were heterozygous for a single *WNT10A* mutation (table 6.1; also see supplementary table 6.1).
All mutations identified were interpreted as potentially damaging. Genealogy showed that the probands carrying an identical *WNT10A* mutation were not related. No consanguinity was found in patients homozygous for an identified *WNT10A* mutation.

Heterozygosity for a mutation in *PAX9* was identified in three patients (p.Y60*, p.Y143C and p.S49L, respectively). In one of the probands, a probably pathogenic *MSX1* mutation (p.R223L) was detected. One patient showed a non-sense mutation in *AXIN2* (p.R656*). In comparison, in 13 syndromic hypodontia cases (76%), mutations were identified of which a *WNT10A* mutation was present in 12 cases (71%) (table 6.1; also see supplementary table 6. 2). One patient showed a *WNT10A* mutation in addition to a pathogenetic *EDA1* mutation that was previously reported in X-linked hypohidrotic ED (OMIM 305100).
The most frequent mutation, p.F228I, represents 62% of the identified *WNT10A* mutations in the non-syndromic hypodontia cohort. This frequency is significantly (OR 17.9, $p<0.05$) higher than the frequency (2.3%) observed in the control population. The hypodontia status of these anonymous controls is not known.

Phenotype of *WNT10A* probands

In six non-syndromic hypodontia patients showing a *WNT10A* mutation, extra-oral symptoms were present. These were considered to be very mild, being part of the normal variation in the population (table 6.1; supplementary table 6.1).
Characteristic features of OODD, including facial telangiectases, evident palmoplantar keratoderma and smooth tongue were not observed. In the syndromic *WNT10A* cases, the most frequent additional features were sparse hair, sparse eyebrows, short eyelashes and abnormalities of the toenails. A dry skin was present in several cases (table 6.1 and supplementary table 6.2).

Table 6.1 Clinical symptoms and genotype in 19 non-syndromic and 11 syndromic hypodontia patients with *WNT10A* mutations

						Teeth
	Proband	**Genotype**	**Gender**	**Age**	**Family History***	**Number Missing Teeth**
	1	p.[C107*]+[=]	f	22	+	16
Non-	2	p.[C107*]+[=]	m	39	+	15
syndromic	3	p.[R128Q]+[=]	f	19	+	20
	4	p.[R163W]+[=]	f	11	+	12
	5	p.[F228I]+[=]	f	28	–	10
	6	p.[F228I]+[=]	f	32	+	14
	7	p.[N306K]+[=]	m	18	–	13
	8	p.[G95K]+[F228I]	m	14	–	28
	9	p.[C107*]+[F228I]	m	10	–	14
	10	p.[C107*]+[F228I]	m	14	+	26
	11	p.[C107*]+[F228I]	f	16	+	14
	12	p.[V145M]+[V145M]	m	18	+	26
	13	p.[F228I]+[F228I]	f	13	+	10
	14	p.[F228I]+[F228I]	m	12	+	17
	15	p.[F228I]+[F228I]	f	15	+	13
	16	p.[F228I]+[F228I]	f	15	–	10
	17	p.[F228I]+[F228I]	m	18	+	11
	18	p.[F228I]+[F228I]	m	19	+	15
	19	p.[F228I]+[F228I]	f	29	+	12
Syndromic	1	p.[C107*]+[=]	m	9	+	12
	2	p.[C107*]+[=]	m	22	+	13
	3	p.[F228I]+[=]	f	34	–	11
	4	p.[F228I]+[=]	m	45	+	18
	5	p.[C107*]+[C107*]	f	7	–	30
	6	p.[C107*]+[F228I]	m	8	+	18
	7	p.[C107*]+[F228I]	f	12	+	20
	8	p.[C107*]+[F228I]	m	15	+	6
	9	p.[F228I]+[F228I]	m	8	+	16
	10	p.[F228I]+[F228I]	f	45	–	14
	11	p.[F228I]+[W277C]	m	11	+	12

+: present; - absent; ±: very mild; Am: male alopecia; Ab: abnormal hair structure, E: Eczema, * family members with tooth agenesis

Table 6.1

Teeth	Hair		Skin			Nails
Abnormal Shape	Scalp	Eyebrows	Dry skin	Hypohidrosis	Plantar hyperkeratosis	
+	–	–	–	–	–	–
+	Am	–	–	–	±	–
+	±	–	–	–	–	–
–	–	–	–	–	–	–
+	–	–	–	–	–	–
–	–	–	–	–	–	–
+	–	–	E	–	–	–
+	–	–	–	–	–	–
+	–	–	–	–	–	–
+	–	–	–	–	–	–
+	–	–	±	–	–	–
+	–	–	–	–	–	–
+	–	–	–	–	–	–
+	–	–	–	–	–	–
+	–	–	–	–	–	–
+	–	–	–	–	–	–
–	–	–	–	–	–	–
+	–	–	–	–	–	–
+	–	–	–	–	–	–
+	–	–	E	–	–	+
–	–	–	+	–	+	–
–	+	–	–	–	–	–
+	+	+	–	+	–	±
+	+	+	+	–	–	+
+	+	+	+	–	–	–
+	–	+	–	–	–	–
+	–	–	±	–	+	+
?	–	–	+	–	–	+
?	+	–	±	–	–	–
+	Ab	–	±	±	–	–

Dental characteristics in *WNT10A* mutation cases

The dental numerical characteristics are presented and the tooth agenesis code (TAC) is calculated (supplementary tables 6.3 and 6.4). The TAC is a unique number that is consistent with a specific pattern of tooth agenesis. [14, 15] No specific TAC could be observed for *WNT10A* mutation carriers. Third molars are seldom present in the current panel. The percentages of tooth agenesis per tooth type are quite similar to those from a larger population of non-syndromic oligodontia patients. [15] The symmetry in agenesis patterns between the left and right sides of the jaw was in line with the population of non-syndromic hypodontia and seen in 58% and 63% of all non-syndromic *WNT10A* cases for the maxilla and the mandible, respectively. In the syndromic *WNT10A* cases, this symmetry for the maxilla and mandible was observed in 46% and 64% of the cases, respectively. Neither the patterns of missing teeth that would distinguish the current population from a general population of non-syndromic hypodontia patients, nor the peculiarities of tooth morphology were observed. The third molar and the upper second premolar were most frequently absent.

Genotype-phenotype

The mean number of missing teeth for the non-syndromic and syndromic *WNT10A* probands was similar, at 15.6 (range:10–28) and 15.4 (range:6–30), respectively (table 6.1 and supplementary tables 6.1 and 6.2). The highest number of missing teeth (30) was present in a p.C107* homozygous girl; an almost complete absence of the permanent dentition was seen and furthermore, also nail dysplasia and mild, sparse curly hair was observed (syndromic patient 5; table 6.1; supplementary table 6.2). The mildest hypodontia, with an agenesis of six teeth, was present in a syndromic patient compound heterozygous for p.C107* and p.F228I (syndromic patient 8; table 6.1; supplementary table 6.2). The absence of more than 20 teeth was observed in patients who were either homozygous, compound heterozygous or heterozygous for *WNT10A* mutations. The patterns of missing teeth did not differ markedly for the *WNT10A* mutations.

Variability of extra-oral features is observed in carriers of a *WNT10A* mutation. Patients with and without additional ectodermal features could be either heterozygous for p.C107*, heterozygous or homozygous for p.F228I. A patient compound heterozygous for p.C107* and p.F228I showed significant features suggestive for an ED (syndromic patient 8; table 6.1; supplementary table 6.2). A patient with the same genotype did not show additional ectodermal features (non-syndromic patient 10; table 6.1; supplementary table 6.1). A patient carrying the p.C107* mutation had an orofacial cleft (syndromic patient 2; table 6.1; supplementary table 6.2).

Family members

To gain more insight into the phenotypic variability of the *WNT10A* mutation within families, family members of patients with a *WNT10A* mutation were studied (supplementary tables 6.5 and 6.6). Tooth agenesis was frequently observed in family members of non-syndromic and syndromic *WNT10A* cases. Sparse hair was most frequently reported in family members of syndromic *WNT10A* cases.

Discussion

This study shows a surprisingly high frequency of *WNT10A* mutations in isolated hypodontia. In 19 out of 34 patients with apparent isolated hypodontia (56%), a mutation in *WNT10A* could be identified. In five probands, a mutation was identified in the candidate genes *MSX1* (one proband), *PAX9* (three probands), *AXIN2* (one proband), respectively. No mutations were found in the *IRF6* gene.

A diagnosis of isolated hypodontia is not easily made. Individuals with ED show variations in phenotypic expression that may range from prominent to very subtle ectodermal symptoms. [3 4 16–18] The latter can be difficult to classify and might hint at features of ED or normal variations. Moreover, hypoplasia of lingual papillae, considered as a characteristic feature in *WNT10A* mutation carriers is difficult to identify. [9, 11] Standard methods of imaging of the tongue papillae are non-invasive video microscopy, contact endoscopy or digital camera after staining with methylene blue. [19–22] However, these are not routinely performed or available in daily clinical practice, and so, were not applied in this study.

After careful examination of our patients, 67% of them were finally classified as non-syndromic. This percentage corresponds with previous studies. [4, 16] Bergendal et al (2006) showed that 14.7% of the oligodontia patients had impaired function of hair, nails and/or sweat glands,3 which is considerably lower than in the studies performed in tertiary centres. [4, 16]

The p.F228I mutation in *WNT10A* was found in normal controls with an allele frequency of 2.3%. This frequency corresponds with the high prevalence of tooth agenesis in the general population. Based on the assumption that heterozygosity for *WNT10A* is involved in 50% of less severe dental agenesis, the expected prevalence of dental agenesis in the Dutch population is approximately 5%. This is in line with the observation that in the European population, 5.5% fail to develop one or more permanent teeth, excluding the

third molar. [1, 2] According to the Hardy and Weinberg rules, and considering an allele frequency of the p.F228I of 1/45, nearly 1 out of 2000 individuals might have a severe hypodontia due to homozygosity for p.F228I. This is approximately half the prevalence (0.14%) of severe hypodontia reported in the European population.

A mutation screen of *MSX1*, *AXIN2*, *PAX9* and the ED genes *EDA*, *EDAR* and *EDARADD* in a population with severe isolated hypodontia revealed a mutation in approximately 11% of the probands. [23]

By including the *WNT10A* gene in the DNA testing, the detection rate of the genetic cause of apparently isolated hypodontia increases to approximately 70% (this study). Data obtained in mice support the involvement of *WNT10* like *MSX1*, *PAX9* and *AXIN2* in tooth development. [24–26] *Wnt10A* is strongly expressed in the dental epithelium at the tooth initiation stage. [25 ,26] *Wnt10A*, as well as *Msx1* and *Pax9*, are also required for normal tooth development beyond the bud stage. [26] *Axin2* is expressed during tooth development in the dental mesenchyme, enamel knot and odontoblasts. [27, 28]

Genotype–phenotype correlations *WNT10A*

Heterozygosity, compound heterozygosity and homozygosity can be responsible for severe hypodontia. Homozygosity, for a non-sense mutation, seems to be associated with an almost complete absence of the permanent dentition. We did not observe a specific pattern of missing teeth in the population carrying a *WNT10A* mutation.

A sex-influenced expression of hypodontia in heterozygotes for a *WNT10A* mutation, as previously suggested by Bohring et al., [11] could also not be confirmed in our study.

Because heterozygosity and compound heterozygosity or homozygosity for *WNT10A* mutations are associated with tooth agenesis, pseudodominant or multigenic patterns of inheritance cannot be excluded.

No relation between the presence or absence of ectodermal features and the specific type of mutation and/or the heterozygous or homozygous state has been detected. In our patient panel, there were less additional ectodermal features compared with previously reported patients. [9, 11, 12] This may reflect a selection bias, but also indicates that other factors, for example, additional genetic factors, may play a role in the phenotypic expression of *WNT10A* mutations. Further study is needed to determine involvement of other factors.

Therefore, we conclude that there is no unambiguous relationship between *WNT10A* genotype and the number of missing teeth, pattern of tooth agenesis and the presence of additional features.

DNA diagnostics in hypodontia patients

To identify the genetic cause in probands with an agenesis of more than six teeth, excluding the third molar, and in probands with a lower number of agenesis with a positive family history, we recommend to test for mutations in *WNT10A*, and if negative to continue with testing for *MSX1*, *PAX9* and *AXIN2*. In case of *AXIN2* mutation analysis, one should specifically ask for possible hereditary colon cancer in the family. Physical examination with focus on additional ectodermal features is of importance. Analysis of *EDA*, *EDAR* and *EDARADD* should be considered in all cases with non-syndromic tooth agenesis of more than six teeth. This approach will improve counselling of patients with hypodontia and their family members.

Acknowledgements

The authors wish to thank all the patients and family members who participated in this study. The authors also thank Nine Knoers for critical reading of the manuscript and helpful discussions.

References

1. Polder BJ, Van't Hof MA, Van der Linden FP, Kuijpers-Jagtman AM. A meta-analysis of the prevalence of dental agenesis of permanent teeth. Community Dent Oral Epidemiol 2004;32:217–26.
2. Nieminen P. Genetic basis of tooth agenesis. J Exp Zool B Mol Dev Evol 2009;312B:320–42.
3. Bergendal B, Norderyd J, Bågesund M, Holst AB. Signs and symptoms from ectodermal organs in young Swedish individuals with oligodontia. Int J Paediatr Dent 2006;16:320–6.
4. Schalk-van der Weide Y, Beemer FA, Faber JA, Bosman F. Symptomalogy of patients with oligodontia. J Oral Rehabil 1994;21:247–61.
5. Bailleul-Forestier I, Molla M, Verloes A, Berdal A. The genetic basis of inherited anomalies of the teeth. Part 1: clinical and molecular aspects of non-syndromic dental disorders. Eur J Med Genet 2008;51:273–91.
6. Bailleul-Forestier I, Berdal A, Vinckier F, de Ravel T, Fryns JP, Verloes A. The genetic basis of inherited anomalies of the teeth. Part 2: syndromes with significant dental involvement. Eur J Med Genet 2008;51:383–408.
7. Visinoni AF, Lisboa-Costa T, Pagnan NA, Chautard-Freire-Maia EA. Ectodermal dysplasias: clinical and molecular review. Am J Med Genet A 2009;149A:1980–2002.
8. Wright JT, Morris C, Clements SE, D'Souza R, Gaide O, Mikkola M, Zonana J. Classifying ectodermal dysplasias: incorporating the molecular basis and pathways (Workshop II). Am J Med Genet A 2009;149A:2062–7.
9. Adaimy L, Chouery E, Megarbane H, Mroueh S, Delague V, Nicolas E, Belguith H, de Mazancourt P, Megarbane A. Mutation in WNT10A is associated with an autosomal recessive ectodermal dysplasia: the odonto-onycho-dermal dysplasia. Am J Hum Genet 2007;81:821–8.
10. Nawaz S, Klar J, Wajid M, Aslam M, Tariq M, Schuster J, Baig SM, Dahl N. WNT10A missense mutation associated with a complete odonto-onycho-dermal dysplasia syndrome. Eur J Hum Genet 2009;17:1600–5.
11. Bohring A, Stamm T, Spaich C, Haase C, Spree K, Hehr U, Hoffmann M, Ledig S, Sel S, Wieacker P, Röpke A. WNT10A mutations are a frequent cause of a broad spectrum of ectodermal dysplasias with sex-biased manifestation pattern in heterozygotes. Am J Hum Genet 2009;85:97–105
12. Cluzeau C, Hadj-Rabia S, Jambou M, Mansour S, Guigue P, Masmoudi S, Bal E, Chassaing N, Vincent MC, Viot G, Clauss F, Manière MC, Toupenay S, Le Merrer M, Lyonnet S, Cormier-Daire V, Amiel J, Faivre L, de Prost Y, Munnich A, Bonnefont JP, Bodemer C, Smahi A. Only four genes (EDA1, EDAR, EDARADD, and WNT10A) account for 90% of hypohidrotic/anhidrotic ectodermal dysplasia cases. Hum Mutat 2011;32:70–2.
13. Kantaputra P, Sripathomsawat W. WNT10A and isolated hypodontia. Am J Med Genet A 2011;155A:1119–22.
14. van Wijk AJ, Tan SP. A numeric code for identifying patterns of human tooth agenesis: a new approach. Eur J Oral Sci 2006;114:97–101.
15. Creton MA, Cune MS, Verhoeven JW, Meijer GJ. Patterns of missing teeth in a population of oligodontia patients. Int J Prosthodont 2007;20:409–13.
16. Nordgarden H, Jensen JL, Storhaug K. Oligodontia is associated with extra-oral ectodermal symptoms and low whole salivary flow rates. Oral Dis 2001;7:226–32.

17. Itin PH. Rationale and background as basis for a new classification of the ectodermal dysplasias. Am J Med Genet A 2009;149A:1973–6.
18. Irvine AD. Towards a unified classification of the ectodermal dysplasias: opportunities outweigh challenges. Am J Med Genet A 2009;149A:1970–2.
19. Miller IJ Jr.. Variation in human fungiform taste bud densities among regions and subjects. Anat Rec 1986;216:474–82.
20. Shahbake M, Hutchinson I, Laing DG, Jinks AL. Rapid quantitative assessment of fungiform papillae density in the human tongue. Brain Res 2005;105:196–201.
21. Just T, Pau HW, Witt M, Hummel T. Contact endoscopic comparison of morphology of human fungiform papillae of healthy subjects and patients with transected chorda tympani nerve. Laryngoscope 2006;116:1216–22.
22. Segovia C, Hutchinson I, Laing DG, Jinks AL. A quantitative study of fungiform papillae and taste pore density in adults and children. Brain Res Dev Brain Res 2002;138:135–46.
23. Bergendal B, Klar J, Stecksén-Blicks C, Norderyd J, Dahl N. Isolated oligodontia associated with mutations in EDARADD, AXIN2, MSX1, and PAX9 genes. Am J Med Genet A 2011;155:1616–22.
24. Klein OD, Minowada G, Peterkova R, Kangas A, Yu BD, Lesot H, Peterka M, Jernvall J, Martin GR. Sprouty genes control diastema tooth development via bidirectional antagonism of epithelial-mesenchymal FGF signaling. Dev Cell 2006;11:181–90.
25. Chen J, Lan Y, Baek JA, Gao Y, Jiang R. Wnt/beta-catenin signaling plays an essential role in activation of odontogenic mesenchyme during early tooth development. Dev Biol 2009;334:174–85.
26. Fujimori S, Novak H, Weissenböck M, Jussila M, Gonçalves A, Zeller R, Galloway J, Thesleff I, Hartmann C. Wnt/β-catenin signaling in the dental mesenchyme regulates incisor development by regulating Bmp4. Dev Biol 2010;348:97–106.
27. Callahan N, Modesto A, Meira R, Seymen F, Patir A, Vieira AR. Axis inhibition protein 2 (AXIN2) polymorphisms and tooth agenesis. Arch Oral Biol 2009;54:45–9.
28. Matalova E, Fleischmannova J, Sharpe PT, Tucker AS. Tooth agenesis: from molecular genetics to molecular dentistry. J Dent Res 2008;87:617–23.

Supplementary table 6.1 Clinical symptoms and Mutational results in 19 *non-syndromic* hypodontia Patients *with WNT10A* Mutations

Patient	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Gender	**f**	**m**	**f**	**f**	**f**	**f**	**m**	**m**
Age (years)	**22**	**39**	**19**	**11**	**28**	**32**	**18**	**14**
Primary teeth abnormal	–	–	+	–	–	–	–	–
Permanent teeth missing	+	+	+	+	+	+	+	+
Number tooth agenesis	16	15	20	12	10	14	13	28
Abnormal shape teeth	+	+	+	–	+	–	+	+
Smooth tongue	–	–	–	–	–	–	–	
Sparse scalp hair	–	Am	±	–	–	–	–	–
Sparse body hair	–	–	–	–	–	–	–	–
Sparse eyebrows	–	–	–	–	–	–	–	–
Short eyelashes	–	–	–	–	–	–	–	–
Hypohidrosis	–	–	–	–	–	–	–	–
Hyperhidrosis	–	–	–	–	–	–	–	–
Dry skin	–	–	–	–	–	–	E	–
Soft, thin skin	–	–	–	–	–	–	–	–
Palmar hyperkeratosis	–	–	–	–	–	–	–	–
Hyperkeratosis on dorsal hands	–	–	–	–	–	–	–	–
Plantar hyperkeratosis	–	±	–	–	–	–	–	–
Palmoplantar sudation	–	±	–	+	–	–	±	–
Dyshidrotic blistering	–	–	–	–	–	–	–	–
Dystrophic fingernails	–	–	–	–	–	–	–	–
Dystrophic toenails	–	–	–	–	–	–	–	–
Photophobia	–	–	–	–	–	–	–	–
Lid cysts	–	–	–	–	–	–	–	–
Family history *	+	+	+	+	–	+	–	–
Mutational result								
Nucleotide substition	c.321	c.321	c.383	c.487	c.682	c.682	c.918	c.283
(first allele)	C>A	C>A	G>A	T>A	T>A	T>A	C>G	>A
Nucleotide substition	c.=	c.=	c.=	c.=	c.=	c.=	c.=	c.682
(second allele)								T>A
Amino acid substitutions	p.C107*	p.C107*	p.R128Q	p.R163W	p.F228I	p.F228I	p.N306K	p.G95K
	p.=	p.=	p.=	p.=	p.=	p.=	p.=	p.F228I

+: present; - absent; ±: very mild ; Am:male alopecia; E: eczema, F: very fair hair
* family members with tooth agenesis

Table 6.1

9	10	11	12	13	14	15	16	17	18	19
m	m	f	m	f	m	f	f	m	m	f
10	14	16	18	13	12	15	15	18	19	29
–	–	–	–	–	–	–	–	–	–	–
+	+	+	+	+	+	+	+	+	+	+
14	26	14	26	10	17	13	10	11	15	12
+	+	+	+	+	+	+	+	–	+	+
–	–	–	–	–	–	–	–	–	–	–
– F	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	±	–
–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–
–	–	±	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–
–	–	–	–	–	–	–	–	–	–	–
–	+	+	+	+	+	+	–	+	+	+
c.321 C>A c.682 T>A p.C107* p.F228I	c.321 C>A c.682 T>A p.C107* p.F228I	c.321 C>A c.682 T>A p.C107* p.F228I	c.433 G>A c.433 G>A p.V145M p.V145M	c.682 T>A c.682 T>A p.F228I p.F228I	c.682 T>A c.682 T>A p.F228I p.F228I	c.682 T>A c.682 T>A p.F228I p.F228I	c.682 T>A c.682 T>A p.F228I p.F228I	c.682 T>A c.682 T>A p.F228I p.F228I	c.682 T>A c.682 T>A p.F228I p.F228I	c.682 T>A c.682 T>A p.F228I p.F228I

Supplementary table 6.2 Clinical symptoms and Mutational results in 11 *syndromic* hypodontia Patients with *WNT10A* mutation

Patient	**1**	**2**	**3**	**4**
Gender	**m**	**m**	**f**	**m**
Age (years)	**9**	**22**	**34**	**45**
Primary teeth abnormal	+	–	–	–
Permanent teeth missing	+	+	+	+
Tooth agenesis (number)	12	13	11	18
Abnormal tooth shape	+	–	–	+
Short roots	–	+	+	–
Smooth tongue	–	–	–	–
Sparse scalp hair	–	–	+	+
Sparse body hair	–	–	–	+
Sparse eyebrows	–	–	–	+
Short eyelashes	–	–	–	+
Hypohidrosis	–	–	–	+
Hyperhidrosis	–	–	–	–
Dry skin	E	+	–	–
Soft, thin skin	–	–	–	–
Palmar hyperkeratosis	–	–	–	–
Hyperkeratosis on dorsal hands	–	–	–	–
Plantar hyperkeratosis	–	+	–	–
Palmoplantar sudation	–	+	–	–
Dyshidrotic blistering	–	–	–	–
Dystrophic fingernails	–	–	–	±
Dystrophic toenails	+	–	–	±
Photophobia	–	–	–	–
Lid cysts	–	–	–	–
Lacrimal duct stenosis	–	–	–	–
Hearing loss	–	–	–	–
Cleft	–	+	–	–
Family history *	+	+	–	+
Mutational result				
nucleotide substition	c.321	c.321	c.682	c.682
(first allele)	C>A	C>A	T>A	T>A
nucleotide substition	c.=	c.=	c.=	c.=
(second allele)				
Amino acid substitutions	p.C107*	p.C107*	p.F228I	p.F228I
	p.=	p.=	p.=	p.=

+: present; -:absent; ±: very mild; E: eczema, Ab: abnormal hair structure
* family members with tooth agenesis

Table 6.2

5	6	7	8	9	10	11
f	m	f	m	m	f	m
7	8	12	15	8	45	11
–	–	–	–	–	–	–
+	+	+	+	+	+	+
30	18	20	6	16	14	12
+	+	+	+	?	+	+
+	?	+	–	?	–	+
–	–	–	±	–	–	–
+	+	–	–	–	+	Ab
–	–	–	–	–	–	–
+	+	+	–	–	–	–
+	+	+	±	–	–	–
–	–	–	–	–	–	±
–	–	–	–	–	–	–
+	+	–	±	+	±	±
–	–	–	–	–	–	–
–	–	–	–	–	–	–
–	–	–	–	±	–	±
–	–	–	+	–	–	–
–	–	–	+	–	–	±
–	–	–	–	–	–	–
+	–	–	–	–	–	–
+	–	–	+	+	–	–
–	–	–	–	–	–	–
–	–	–	–	–	–	–
–	–	–	–	–	–	–
–	–	–	–	–	–	–
–	–	–	–	–	–	–
–	+	+	+	+	–	+
c.321	c.321	c.321	c.321	c.682	c.682	c.682
C>A	C>A	C>A	C>A	T>A	T>A	T>A
c.321	c.682	c.682	c.682	c.682	c.682	c.831
C>A	T>A	T>A	T>A	T>A	T>A	G>C
p.C107*	p.C107*	p.C107*	p.C107*	p.F228I	p.F228I	p.F228I
p.C107*	p.F228I	p.F228I	p.F228I	p.F228I	p.F228I	p.W277C

Supplementary table 6.3 Tooth findings in 19 *non-syndromic* hypodontia Patients with *WNT10A* Mutations

							upper right		
							94,70%	42,10%	0,00%
Patient	**Genotype**	**Age**	**Gender**	**TACoverall**	**Number of missing teeth**	**TAC 1st quadrant**	**18**	**17**	**16**
1	p.[C107*]+[=]	22	f	210.210.209.209	16	210	x	x	
2	p.[C107*]+[=]	39	m	158.158.144.152	15	158	x		
3	p.[R128Q]+[=]	19	f	158.158.159.153	20	158	x		
4	p.[R163W]+[=]	11	f	142.142.144.144	12	142	x		
5	p.[F228I]+[=]	28	f	16.028.152.152	10	16			
6	p.[F228I]+[=]	32	f	208.152.216.216	14	208	x	x	
7	p.[N306K]+[=]	18	m	216.216.144.112	13	216	x	x	
8	p.[G95K]+[F228I]	14	m	222.252.255.255	28	222	x	x	
9	p.[C107*]+[F228I]	10	m	192.192.219.209	14	192	x	x	
10	p.[C107*]+[F228I]	14	m	222.222.223.223	26	222	x	x	
11	p.[C107*]+[F228I]	16	f	138.138.197.197	14	138	x		
12	p.[V145M]+[V145M]	18	m	222.222.223.223	26	222	x	x	
13	p.[F228I]+[F228I]	13	f	146.146.144.144	10	146	x		
14	p.[F228I]+[F228I]	12	m	150.158.135.135	17	150	x		
15	p.[F228I]+[F228I]	15	f	158.156.144.144	13	158	x		
16	p.[F228I]+[F228I]	15	f	200.204.192.128	10	200	x	x	
17	p.[F228I]+[F228I]	18	m	130.146.152.152	11	130	x		
18	p.[F228I]+[F228I]	19	m	152.152.153.217	15	152	x		
19	p.[F228I]+[F228I]	29	f	152.216.208.144	12	152	x		
19	p.[F228I]+[F228I]	29	f		12	144	x		
18	p.[F228I]+[F228I]	19	m		15	217	x	x	
17	p.[F228I]+[F228I]	18	m		11	152	x		
16	p.[F228I]+[F228I]	15	f		10	128	x		
15	p.[F228I]+[F228I]	15	f		13	144	x		
14	p.[F228I]+[F228I]	12	m		17	135	x		
13	p.[F228I]+[F228I]	13	f		10	144	x		
12	p.[V145M]+[V145M]	18	m		26	223	x	x	
11	p.[C107*]+[F228I]	16	f		14	197	x	x	
10	p.[C107*]+[F228I]	14	m		26	223	x	x	
9	p.[C107*]+[F228I]	10	m		14	209	x	x	
8	p.[G95K]+[F228I]	14	m		26	255	x	x	x
7	p.[N306K]+[=]	18	m		13	112		x	x
6	p.[F228I]+[=]	32	f		14	216	x	x	
5	p.[F228I]+[=]	28	f		10	152	x		
4	p.[R163W]+[=]	11	f		12	144	x		
3	p.[R128Q]+[=]	19	f		20	153	x		
2	p.[C107*]+[=]	39	m		15	152	x		
1	p.[C107*]+[=]	22	f		16	209	x	x	
					Number of missing teeth	**TAC 4th quadrant**	**48**	**47**	**46**
							94,70%	47,40%	10,50%
f, female; m, male, TAC, ; Tooth Agenesis Code; x, absent teeth							**lower right**		

Table 6.3

upper right					upper left								
73,70%	63,20%	42,10%	63,20%	0,00%	0,00%	52,60%	52,60%	78,90%	78,90%	5,30%	42,10%	94,70%	
15	**14**	**13**	**12**	**11**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**TAC 2nd quadrant**
x			x			x			x		x	x	210
x	x	x	x			x	x	x	x			x	158
x	x	x	x			x	x	x	x			x	158
	x	x	x			x	x	x				x	142
x							x	x	x				28
x								x	x			x	152
x	x							x	x		x	x	216
x	x	x	x				x	x	x	x	x	x	252
											x	x	192
x	x	x	x			x	x	x	x		x	x	222
	x		x			x		x				x	138
x	x	x	x			x	x	x	x		x	x	222
x			x			x			x			x	146
x		x	x			x	x	x	x			x	158
x	x	x	x				x	x	x			x	156
	x						x	x			x	x	204
			x			x			x			x	146
x	x							x	x			x	152
x	x							x	x		x	x	216
x									x		x	x	208
x	x			x	x			x	x			x	153
x	x							x	x			x	152
											x	x	192
x									x			x	144
		x	x	x	x	x	x					x	135
x									x			x	144
x	x	x	x	x	x	x	x	x	x		x	x	223
		x		x	x		x				x	x	197
x	x	x	x	x	x	x	x	x	x		x	x	223
x				x	x	x		x	x		x	x	219
x	x	x	x	x	x	x	x	x	x	x	x	x	255
x									x			x	144
x	x							x	x		x	x	216
x	x							x	x			x	152
x									x			x	144
x	x			x	x	x	x	x	x			x	159
x	x								x			x	144
x				x	x				x		x	x	209
45	**44**	**43**	**42**	**41**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**	**TAC 3rd quadrant**
84,20%	47,40%	26,30%	21,10%	47,40%	47,40%	31,60%	31,60%	47,40%	84,20%	5,30%	47,40%	100,00%	
lower right					lower right								

Supplementary table 6.4 Tooth findings in 11 *syndromic* hypodontia Patients *with WNT10A* Mutations

								upper right		
							100,00%	45,50%	9,10%	
Patient	**Genotype**	**Age**	**Gender**	**TACoverall**	**Number of missing teeth**	**TAC 1st quadrant**	**18**	**17**	**16**	
1	p.[C107*]+[=]	9	m	146.158.144.144	12	146	x			
2	p.[C107*]+[=]	22	m	144.155.145.145	13	144	x			
3	p.[F228I]+[=]	34	f	216.216.192.145	11	216	x	x		
4	p.[F228I]+[=]	45	m	154.154.217.217	18	154	x			
5	p.[C107*]+[C107*]	7	f	254.254.255.255	30	254	x	x	x	
6	p.[C107*]+[F228I]	8	m	198.194.211.219	18	198	x	x		
7	p.[C107*]+[F228I]	12	f	214.218.211.199	20	214	x	x		
8	p.[C107*]+[F228I]	15	m	130.130.128.128	6	130	x			
9	p.[F228I]+[F228I]	8	m	152.154.217.153	16	152	x			
10	p.[F228I]+[F228I]	45	f	208.208.209.209	14	208	x	x		
11	p.[F228I]+[W277C]	11	m	146.124.065.065	12	146	x			
11	p.[F228I]+[W277C]	11	m		12	65		x		
10	p.[F228I]+[F228I]	45	f		14	209	x	x		
9	p.[F228I]+[F228I]	8	m		16	153	x			
8	p.[C107*]+[F228I]	15	m		6	128	x			
7	p.[C107*]+[F228I]	12	f		20	199	x	x		
6	p.[C107*]+[F228I]	8	m		18	219	x	x		
5	p.[C107*]+[C107*]	7	f		30	255	x	x	x	
4	p.[F228I]+[=]	45	m		18	217	x	x		
3	p.[F228I]+[=]	19	f		11	128	x			
2	p.[C107*]+[=]	22	m		13	145	x			
1	p.[C107*]+[=]	9	m		12	144	x			
					Number of missing teeth	**TAC 4th quadrant**	**48**	**47**	**46**	
							90,90%	54,50%	9,10%	
f, female; m, male, TAC, ; Tooth Agenesis Code; x, absent teeth								**lower right**		

Table 6.4

upper right					upper left								
81,80%	36,40%	27,30%	63,60%	0,00%	9,10%	72,70%	27,30%	72,70%	81,80%	18,20%	54,50%	90,90%	
15	**14**	**13**	**12**	**11**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**TAC 2nd quadrant**
x			x			x	x	x	x			x	158
x					x	x		x	x			x	155
x	x							x	x		x	x	216
x	x		x			x		x	x			x	154
x	x	x	x			x	x	x	x	x	x	x	254
		x	x			x					x	x	194
x		x	x			x		x	x		x	x	218
			x			x						x	130
x	x					x		x	x			x	154
x									x		x	x	208
x			x				x	x	x	x	x		124
				x	x						x		65
x				x	x				x		x	x	209
x	x			x	x			x	x		x	x	217
												x	128
		x	x	x	x	x			x		x	x	211
x	x		x	x	x	x			x		x	x	211
x	x	x	x	x	x	x	x	x	x	x	x	x	255
x	x			x	x			x	x		x	x	217
											x	x	192
x				x	x				x			x	145
x									x			x	144
45	**44**	**43**	**42**	**41**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**	**TAC 3rd quadrant**
63,60%	36,40%	18,20%	27,30%	72,70%	72,70%	27,30%	9,10%	27,30%	72,70%	9,10%	72,70%	90,90%	
lower right					lower right								

Table 6.5

Supplementary table 6.5 Clinical Manifestations in Family members of patients with *non-syndromic* hypodontia and *WNT10A* mutation

Proband	Age; Gender Genotype Number missing teeth	Family members	Genotype family members	Affected Structures teeth	nails	skin	hair	hypohidrosis
1	22 yr; f	father	p.[C107*]+[=]	M	–	–	Am	–
	p.[C107*]+[=]	mother	[=]+[=]	Ex	–	–	Gr	–
	16	brother	[=]+[=]	Ex	–	–	–	–
2	39 yr; m	father		–	–	–	–	–
	p.[C107*]+[=]	mother		–	–	–	Gr	–
	15	son		M	–	–	–	–
		sister		M	–	–	–	–
3	19 yr; f	father	p.[R128Q]+[=]	M	–	–	Am	–
	p.[R128Q]+[=]	mother	[=]+[=]	–	–	–	–	–
	20	SP		T				
		SP		T				
4	11 yr; f	father		M	–	–	–	–
	p.[R163W]+[=]	mother		M	–	–	–	–/Ps
	12	SF		M				
		SM		M				
5	28 yr; f	father		–	–	–	–	–
	p.[F228I]+[=]	mother		–	–	–	–	–
	10							
6	32 yr; f	father		–	–	–	–	–
	p.[F228I]+[=]	mother		M	–	–	–	–
	14	son		M	–	–	–	–
7	18 yr; m	father		M3	–	–	–	–
	p.[N306K]+[=]	mother		–	–	–	–	–
	13							
8	14 yr; m	father		Ex	–	–	Am	–
	p.[G95K]+[F228I]	mother		Ex	–	–	–	–
	28				–	–	–	–
9	10 yr; m	father		–	–	–	–	–
	p.[C107*]+[F228I]	mother	p.[F228I]+[F228I]	–	±	–	–	–
	14							
10	14 yr; m	father	p.[F228I]+[=]	M/T?	–	–	Am	–
	p.[C107X]+[F228I]	mother	p.[C107*]+[=]	M3	–	–	Bo	±
	24?	FM		–	–	–	–	–
		MM		M3/M?	–	–	–	–

11	16 yr; f	father		–	–	–	–	–
	p.[C107*]+[F228I]	mother	p.[F228I]+[=]	–	–	D	–	–
	14	sister	p.[C107*]+[F228I]	M	±	D	–	–
12	18 yr, m	father		M			Am	
	p.[V145M]+[V145M]	mother		T			–	
	26	brother		M			–	
13	13 yr; f	father	p.[F228I]+[=]	M3	–	–	–	–
	p.[F228I]+[F228I]	mother	p.[F228I]+[=]	T	–	–	Af	–
	10	brother		M	–	–	–	–
		SM		M				–
14	12 yr; m	father		–	–	–	–	–
	p.[F228I]+[F228I]	mother		–	–	–	–	–
	17	MP		M	–	–	B	+
15	15 yr; f	father	p.[F228I]+[=]	–	–	_	–	–
	p.[F228I]+[F228I]	mother	p.[F228I]+[=]	–	–	–	–	–
	13	sister		T	±	–	–	–
		brother		M				
16	15 yr; f	father		–	–	–	–	–
	p.[F228I]+[F228I]	mother	p.[F228I]+[=]	–	–	–	–	–
	10							
17	18 yr; m	father	p.[F228I]+[=]	–	–	Ec	–	–
	p.[F228I]+[F228I]	mother	p.[F228I]+[=]	–	–	–	S	–
	11	cousin		M	–	–	–	–
18	19 yr; m	father		C	–	–	Am	–
	p.[F228I]+[F228I]	mother		–	–	–	–	–
	15	brother		M	–	–	–	–
		FP		M	–	–	–	–
19	29 yr; f	father	p.[F228I]+[=]	–	–	–	–	–
	p.[F228I]+[F228I]	mother	p.[F228I]+[=]	–	–	–	-	–
	12	sister	p.[F228I]+[F228I]	M	–	–	±	–

Family: SP, sister of father; SM, sister of mother; FP, brother father;
PP, paternal grandfather
+, present; –, absent; ±, mild affected
Teeth: C, cleft; Ex, no data on agenesis due to extractions M, agenesis of 2 to 6 permanent teeth except third molars without further information available; M3, absent third molars; T, single tooth absent, excluding M3; ?, one or two teeth might be extracted;

Skin: D, dry skin; Ec eczema;
Hair: Af, female pattern hair loss, Am, male alopecia; Bo, bold until 2 years of age; B, sparse body hair, Gr, early grey hair; S, sparse scalp hair;
Hypohidrosis: Ps, Palmoplantar sudation
A blank entry indicates no information is available

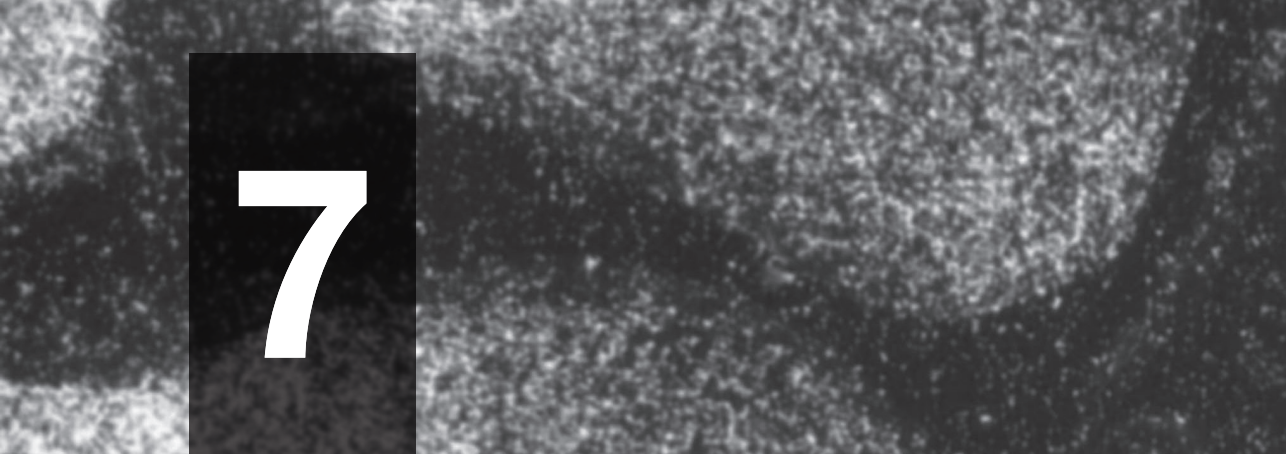
Supplementary table 6.6 Clinical Manifestation in Family members of patients with *syndromic* hypodontia and *WNT10A* mutation

Table 6.6

Proband	Age; Gender Genotype Number missing teeth	Family members	Genotype family members	Affected Structures				
				teeth	nails	skin	hair	hypohidrosis
1	9 yr, m	father		–	–	–	–	–
	p.[C107*]+[=]	mother	p.[C107*]+[=]	M	±	–	Gr	–
	12	brother		B				
2	22 yr, m	father		–	–	–	–	–
	p.[C107*]+[=]	mother		M	–	D	–	–
	13	SM		M	–	–	–	+
3	34 yr, f	father	[=]+[=]	–	–	–	–	–
	p.[F228I]+[=]	mother	p.[F228I]+[=]	Dt*	–	–	S	–
	11							–
4	45 yr, m	father		–	–	–	–	–
	p.[F228I]+[=]	mother		G	–	–	S	±
	18	sister		A	–	–	–	–
		son	p.[F228I]+[=]	M	–	–	–	–
		niece	p.[F228I]+[F228I]	M	I	–	S	–
		nephew		G				
5	7 yr; f	father	p.[C107*]+[=]	–	–	–	–	–
	p.[C107*]+[C107*]	mother	p.[C107*]+[=]	–	–	–	–	–
	30	PM		T	–	Ec #	–	–
		PP		–	–	–	Am*	–
6	8 yr; m	father		–	–	–	Bo	–
	p.[C107*]+[F228I]	mother		–	–	–	–	–
	18	sister	p.[C107*]+[F228I]	M	–	D	E	–
		sister	p.[C107*]+[F228I]	M	–	D	S, E	–
		FM		M			S	
		4 cousins mother		M				
		4 cousins once removed		M				
7	12 yr; f	father		–	–	–	–	–
	p.[C107*]+[F228I]	mother		M3	–	–	–	–
	20	brother	p.[C107*]+[F228I]	M	–	–	E, L, S (±)	–

8	15 yr; m	father	p.[F228I]+[=]	–	–	–	–	–
	p.[C107*]+[F228I]	mother	p.[C107*]+[=]	A	–	D,Ec	–	±
	6	MM				S		±
9	8 yr; m	father	p.[F228I]+[=]	–	–	–	Am*	–
	p.[F228I]+[F228I]	mother	p.[F228I]+[=]	M	–	–	–	–
	16	sister	p.[F228I]+[F228I]	M	–	–	–	–
		MM		M				
10	45 yr; f	father		–	–	–	–	–
	p.[F228I]+[F228I]	mother		–	–	–	S/F	–
	14	sister		–	–	–	S/F	–
11	11 yr; f	father		M3	–	–	–	–
	p.[F228I]+[W277C]	mother		–	–	–	–	–
	12	cousin		A/Sp	–	Ec	Ab	–
		FP		T	–		–	

+, present; - , absent; (±), mild
Teeth: A, markedly small, or abnormal shape or missing upper lateral permanent incisors; Sp, wide spaced teeth
B, agenesis permanent incisors; C, agenesis of upper permanent canines; Dt, dental prostheses; G, permanent teeth are markedly small;
M, agenesis of 2 to 6 permanent teeth except third molars without further information available; M3, absent third molars;
T, single tooth absent, excluding M3;
Nails: I, indentations nails, very mild
Skin: D, dry skin; Ec, eczema;
Hair: Ab: abnormal structure; Am, male alopecia; Bo, bold until 3 years; E, sparse eyebrows F, fragile hair; Gr, early grey hair; L, sparse eyelashes; S, sparse scalp hair
*: At young age; #: Hands, transient
A blank entry indicates that there was no information available



7

Chapter 7

MSX1 and tooth dimensions

Three-dimensional analysis of tooth dimensions in the MSX1-missense mutation.

Créton M, van den Boogaard MJ, Maal T, Verhamme L, Fennis W, Carels C, Kuijpers-Jagtman AM, Cune M.

Published in: Clin Oral Investig. 2012 Aug 31.

Abstract

Objectives

A novel, 3D technique to measure the differences in tooth crown morphology between the *MSX1* cases and non-affected controls was designed to get a better understanding of dental phenotype-genotype associations.

Materials and methods

Eight Dutch subjects from a single family with tooth agenesis, all with an established nonsense mutation c.332 C > A, p.Ser111* in exon 1 of *MSX1*, were compared with unaffected controls regarding several aspects of tooth crown morphology of incisor and molar teeth. A novel method of quantitative three-dimensional analysis was used to detect differences.

Results

Statistically significant shape differences were observed for the maxillary incisor in the *MSX1* family compared with the controls on the following parameters: surface area, buccolingual dimension, squareness, and crown volume ($P \leq 0.002$). Molar crown shape was unaffected.

Conclusions

A better understanding of dental phenotype-genotype associations may contribute to earlier diagnosis of some multiple-anomaly congenital syndromes involving dental anomalies.

Clinical relevance

A "shape database" that includes associated gene mutations resulting from developmental syndromes may facilitate the genetic identification of hypodontia cases.

Introduction

Hypodontia, the congenital absence of one or more permanent teeth, is the most common developmental anomaly in humans, with a prevalence of 5.5 % in Europeans and a 1.37:1 preference for women compared with men. Hypodontia appears as part of a syndrome or as a non-syndromic trait [1-5] and has a heterogeneous dental and dento-facial presentation.

There is an increasing understanding with respect to the genetic background of tooth development and, more specifically, to the molecular mechanisms during cell and tissue interactions. The etiology of disturbances of tooth development is considered multifactorial and genetic, epigenetic, and environmental, and their interaction factors play a role. [6-9] Over 300 genes have been associated with tooth development to date, at least in mouse embryos. Most of these genes seem to have a function in preserved signaling pathways during repetitive and reciprocal cellular communication between epithelial and mesenchymal tissues. [9-12]

When tooth development is disrupted, a wide spectrum of clinical phenotypes can be expected, including typical patterns of tooth agenesis and variations in tooth morphology and size. For example, in familial non-syndromic oligodontia, it has been suggested that the defects in *PAX9* predispose for agenesis of maxillary and mandibular second molars, while in *MSX1*-associated oligodontia, agenesis of bicuspids is typically observed.

More recently, some variations in the *EDA* gene have been demonstrated to cause (X-linked) non-syndromic oligodontia as well. The patterns of missing teeth associated with *EDA* mutations seem to differ distinctly from those observed with *PAX9* and *MSX1*. There appears to be a tendency towards agenesis of maxillary and mandibular central incisors, lateral incisors, and canines in the presence of an *EDA* mutation. In case of *PAX9* or *MSX1* mutations, maxillary and mandibular first preliminary molars frequently persist. [13, 14] In the case of mutations in the *SHH* gene, fusion of the central incisor tooth buds may result in a single central incisor. [15] In addition, subjects with a single missing central upper incisor can be heterozygous carriers for holoprosencephaly [16], a potentially more serious syndromic condition affecting the midline development of the brain and face.

Some examples of aberrations in tooth shape and dimensions that are associated with genetic disturbances are provided below. Mutations in the *EDA* pathway (*EDA*, *EDAR*, *EDARADD*, and *NEMO*) result in hypohydrotic ectodermal dysplasia, commonly with hypodontia and conical, peg-shaped teeth when they do develop. [17, 18] In addition, it has been established that a common variation in *EDAR* (*EDAR* 1540C) in a Japanese population is strongly associated with the degree to which marginal ridges on the lingual surfaces of upper incisors (tooth shoveling) are developed. [19] A typical combination of morphological tooth features is observed in association with a *DLX3* mutation (amelogenesis imperfecta with taurodontism). [20] In cases of hypodontia, teeth that are

formed are generally smaller (microdontia) than those encountered in subjects without tooth agenesis [18, 21-23], while subjects with supernumerary teeth (hyperdontia) generally have larger teeth (macrodontia) than the controls. [23, 24] Recently, mutations in the *PCNT* gene have been shown to be associated with very small teeth, possibly the smallest ever reported. [25] Taurodontia is frequently observed in subjects with hypodontia [26, 27] but not those with hyperdontia. [28] Therefore, a better understanding of dental phenotype–genotype associations may contribute to earlier diagnosis of some multiple congenital anomaly syndromes involving tooth anomalies; additionally, precise measuring tools for shape analysis are desirable. [29]

Morphological tooth traits, parameters of tooth dimension, and agenesis patterns may also serve as biomarkers for a dental phenotype, enabling early diagnosis of syndromes or specific genetic disorders. [18] The National Institutes of Health defines a biomarker as a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention. Biomarkers may be anatomic, physiologic, biochemical, or molecular measures that reflect the presence or severity of specific disease states.

Quantitative morphological analysis of the teeth poses a fundamental problem because teeth are multi-dimensional, irregular objects that are difficult to measure and quantify. [30] Continuous measures are preferred over descriptive ones, such as the presence or absence of Carabelli's trait or hypocone reduction. In odontometric analyses, linear measurements such as the mesiodistal and buccolingual tooth dimensions are traditionally performed on dental casts by means of analogue or digital calipers. This type of measurement can be obtained with a high degree of inter- and intra-observer reliabilities [23]. More recently, two-dimensional (2D) image analysis systems became available, and non-linear measurements, such as surface areas and perimeters, could be reliably determined. [31] Because both mesiodistal and buccolingual dimensions are generally smaller in subjects affected by hypodontia [23], tooth volume is expected to be an even more discriminative three-dimensional (3D) parameter which distinguishes small differences in tooth dimension between subgroups of patients.

For this purpose, we have developed a technique to geometrically evaluate the morphological parameters of teeth in three dimensions. This technique was applied to compare a series of patients with a known *MSX1* mutation with healthy controls under the null hypothesis that they are similar. Observed differences in tooth crown morphology

between the *MSX1* cases and non-affected controls will be discussed in light of the present understanding of the biological regulation regarding some features of tooth crown morphogenesis.

Materials and methods

This study was designed as a case–control study.

Cases

Eight Dutch subjects from one family (four males and four females) with tooth agenesis and cleft palate or cleft lip and palate participated in the study. Subjects' ages varied from 9 to 68 years (mean age, 39 years). All subjects had an established nonsense mutation c.332 C > A, p. Ser111* in exon 1 of *MSX1*. This particular population has been previously described in detail by van den Boogaard et al., and this mutation was known as S105X. The nomenclature has been changed in accord with the guidelines of the Human Genome Variation Society with reference sequence NM_002448.3. [32] Absent teeth are presented in Table 7.1.

Table 7.1 Congenitally absent teeth in the *MSX1* cases

Table 7.1

	Maxilla	18	17	16	15	14	13	12	11	21	22	23	24	25	26	27	28
	Mandible	48	47	46	45	44	43	42	41	31	32	33	34	35	36	37	38
Male																	
1	Maxilla	X			X	X							X	X			X
	Mandible	X			X									X			X
2	Maxilla	X	X		X	X							X	X			X
	Mandible	X			X									X		X	X
3	Maxilla	X			X	X							X	X			X
	Mandible	X			X									X		X	X
4	Maxilla	X			X	X							X	X			X
	Mandible	X	X	X	X									X	X	X	X
Female																	
1	Maxilla	X			X	X							X	X			X
	Mandible	X			X									X			X
2	Maxilla				X	X							X	X			
	Mandible	X			X									X			X
3	Maxilla	X			X	X							X	X			X
	Mandible	X	X	X	X									X	X	X	X
4	Maxilla	X	X		X	X							X	X		X	X
	Mandible	X	X	X	X				X	X				X	X	X	X

X, congenitally missing

Selection of target teeth

Like other families with an *MSX1* mutation, the majority of the family members in the present study lack the premolars, predominantly the second premolars (Table 7.1). [24] Therefore, we selected two tooth types that were present in all family members and, in general, are present in even the most severe hypodontia cases: the right maxillary first molar and the central incisor.

Controls

Healthy Caucasian subjects without hypodontia served as controls (21 males and 21 females). They were selected from the database of the Department of Orthodontics and Craniofacial Biology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands. To be included, all permanent teeth had to be present, including the third molars. The right maxillary central incisor and maxillary first molar should be non-restored and fully erupted. Subjects were excluded when the target teeth were damaged, showed excessive tooth wear, or with severe crowding.

3D measurements of tooth dimensions

Conventional gypsum models were processed into digital dental models, and their raw geometric data were obtained for all cases and controls (Digimodel, OrthoProof B.V., Doorn, The Netherlands). Target teeth were virtually cut from the models using commercially available software (Maxilim, Medicim B.V., Mechelen, Belgium). Subsequently, the teeth were loaded into a computer program that enables the mathematical analysis of three-dimensional shapes (Matlab 2007b, the Mathworks, Natick, MA, USA; Fig. 7.1).

Figure 7.1

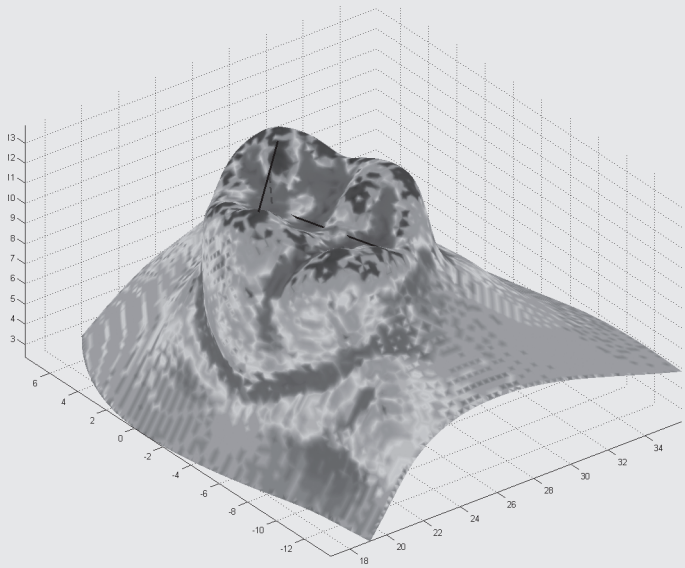


Figure 7.1 The teeth were loaded into a computer program that enables mathematical analysis of three-dimensional shapes. For the first molar, a line is drawn between the two mesial cusp tips. A second line is drawn from the mesial to the distal margin. These two lines are then projected onto each other to form a plane parallel to the occlusal plane.

The teeth must be positioned reproducibly in the geometric model. For this purpose, a reference plane was defined for both the molar and the incisor on the basis of predefined reference points, as described below. All geometric measurements were performed from this reference plane.

Maxillary first molar

For the first molar, a line was drawn between the mesial buccal and lingual cusp tips. A second line was drawn from the mesial to the distal margin (Fig. 7.1). These two lines were projected onto each other to form a plane parallel to the occlusal plane. The molar was then rotated automatically until the constructed plane was parallel to the *X*–*Y* plane (Fig. 7.2).

Figure 7.2

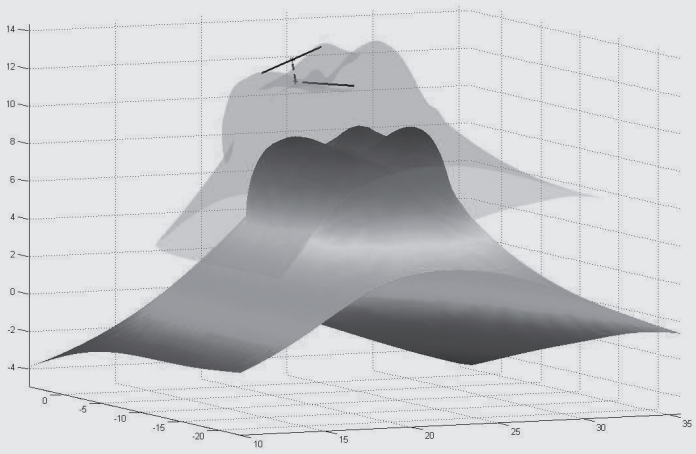


Figure 7.2 The molar is rotated automatically until the constructed plane is parallel to the *X-Y* plane.

The initial idea was to lower the plane until the largest perimeter of the molar was reached. Unfortunately, this often presents sub-gingivally, where no data regarding tooth dimensions are available. Therefore, the plane was lowered 1.2 mm below the deepest point in the fissure (which was determined automatically). At this depth, all models could be included in the study. The position of the reference plane had now been established.

Maxillary central incisor

For the incisor, a line was drawn through the incisal edge. Subsequently, the most prominent point on the buccal surface and the margin of the cingulum on the palatal surface were determined, and lines were drawn through these two points (perpendicular to the incisal edge line). To create a plane, the model was rotated along the incisal edge line until these two newly constructed lines were equal in length (Fig. 7.3). Finally, the reference plane was determined 2.3 mm beneath the incisal edge line. This position was chosen because it ensured that the reference plane would be above the gingival margin in all cases.

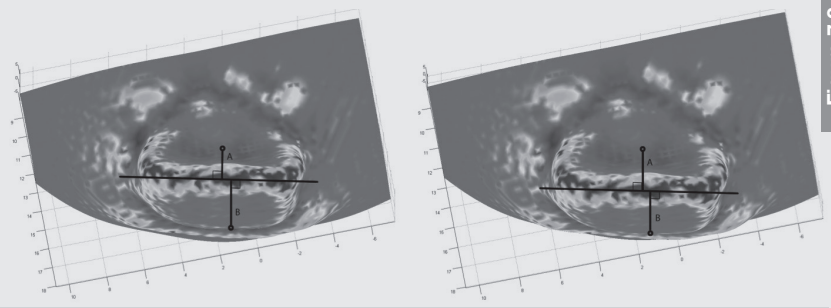


Figure 7.3

Figure 7.3 To create a plane, the model is rotated along the incisal edge line until the two newly constructed lines (A and B) are equal in length. Line A is drawn perpendicular to the incisal edge line, through the center of the cingulum. Line B is also drawn perpendicular to the incisal edge line, through the most prominent buccal point.

Morphological quantitative parameters

Tooth crown shape was quantified by means of six parameters that were thought to be representative, all of which were calculated at the level of the reference plane. These parameters were defined as follows:

- Perimeter: the perimeter of the crown at the level of the reference plane (mm);
- Surface area: the surface of the plane at the level of the reference plane (mm^2);
- Buccolingual distance: the maximal distance between the buccal surface and the lingual surface of the crown (mm);
- Mesiodistal distance: the maximum distance between the mesial and distal proximal surfaces (mm);
- Squareness: indicated to what degree the tooth crown shape was square and was the ratio between the mesiodistal and buccolingual distances (mm/mm);
- Volume: the volume of the crown was calculated from the reference plane to the incisal edge and cusps (mm^3).

Repeatability

Twenty randomly selected incisors and 20 randomly selected molars were measured and remeasured by the same observer, and 20 molars and 20 incisors were measured by another observer to assess intra- and inter-observer repeatabilities. Repeatability of the measurements was expressed as the coefficient of repeatability (CR) in accordance with Bland and Altman. [33]

Statistical analysis

Two-way analysis of variance with the geometric parameters as dependent variables and group and gender as fixed factors was applied for the measurements on both the molar and the incisor. Tooth dimensions are likely to be correlated, and multiple-testing correction to overcome the increase in type I error was advisable; hence, Bonferroni correction was performed. Consequently, α was set at 0.01. This is considered to be a conservative approach.

Results

The CRs for both intra- and inter-observer repeatabilities are presented in Table 7.2. The CR was interpreted in accordance with the guidelines of the British Standards Institution, which states that 95 % of the difference between the first and second measurements is expected to be within two standard deviations of the mean difference. [34] This was the case for the inter-observer repeatability of all measurements on both molars and incisors and was considered satisfactory. Regarding the intra-observer repeatability of the mesiodistal distance, area, and volume, 90 % of the differences fell within two standard deviations of the mean difference, while all other parameters were at 95 % (Table 7.2).

Table 7.2 Intra- and inter-observer repeatabilities for incisors (n = 20) and molars (n = 20) **Table 7.2**

	Coefficient of Repeatability [33]	
	Intra-observer repeatability	Inter-observer repeatability
Incisors		
Perimeter (mm)	0.8	1.1
Mesiodistal distance (mm)	0.3	0.5 *
Buccolingual distance (mm)	0.3	0.3
Area (mm^2)	1.9	1.4 *
Volume (mm^3)	4.5	2.0 *
Molars		
Perimeter (mm)	1.2	3.2
Mesiodistal distance (mm)	1.0	1.4
Buccolingual distance (mm)	0.3	0.6
Area (mm^2)	3.1	6.6
Volume (mm^3)	12.2	12.3

* a total of 90% of differences fall within two standard deviations of the mean difference. With all other coefficients, 95% of differences fall within two standard deviations of the mean difference

The mean values and standard deviations for *MSX1* cases and controls, as well as the statistical comparisons, are presented in Table 7.3. For the first maxillary molar, no statistically significant differences were noted for any of the six parameters nor were interaction effects noticeable between gender and group (*MSX1* case or control). Therefore, the null hypothesis that there are no differences in dimensions of the maxillary right first molar between *MSX1* cases and non-affected controls could not be rejected.

Table 7.3 Descriptive statistics for geometric parameters of crown morphology for molars and incisors in *MSX1* cases and controls.

	Control ($n = 42$) Mean (SD)	Case MSX1 ($n=8$) Mean (SD)	Gender *P* value	Group *P* value	Gender × Group *P* value
Molar					
Perimeter (mm)	35.9 (2.4)	34.7 (4.6)	0.962	0.264	0.199
Area (mm^2)	93.2 (10.7)	89.8 (23.0)	0.733	0.513	0.191
Buccolingual distance (mm)	9.7 (0.5)	9.3 (1.3)	0.673	0.114	0.699
Mesiodistal distance (mm)	10.7 (0.8)	10.2 (1.2)	0.767	0.193	0.962
Squareness (mm/mm)	1.1 (0.1)	1.1 (0.1)	0.688	0.336	0.848
Volume (mm^3)	151.6 (24.5)	152.1 (57.9)	0.708	0.958	0.159
Incisor					
Perimeter (mm)	21.2 (1.7)	22.1 (1.6)	0.096	0.250	0.598
Area (mm^2)	24.3 (3.7)	31.3 (7.5)	0.079	0.000 *	0.484
Buccolingual distance (mm)	3.0 (0.3)	4.2 (0.7)	0.051	0.000 *	0.912
Mesiodistal distance (mm)	8.6 (0.6)	8.7 (0.9)	0.127	0.693	0.732
Squareness (mm/mm)	2.8 (0.3)	1.9 (0.3)	0.380	0.000 *	0.965
Volume (mm^3)	35.8 (5.6)	45.6 (13.1)	0.301	0.002 *	0.986

Mean values and standard deviations (in parentheses). Univariate analysis of variance with geometric parameters as dependent variables and gender and group as fixed factors, *P* values given
* Statistically significant values below $\alpha = 0.01$ (Bonferroni correction)

Regarding the central incisor, the *MSX1* cases have significantly higher values for the area of the reference plane as well as its buccolingual distance, squareness, and volume. Again, there were no interaction effects between gender and group. For the central incisor, the null hypothesis that there are no differences in dimensions between *MSX1* cases and controls can be rejected. Digital models of a typical *MSX1* case and a control are seen in Fig. 7.4 a, b respectively.

Figure 7.4

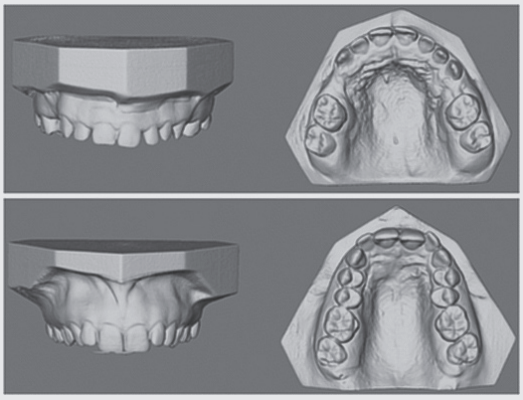


Figure 7.4 a Digital model of the frontal (left) and occlusal (right) views of a typical *MSX1* case (female). It is noticeable that the teeth of the *MSX1* female are distinctive in shape and size compared to a control (i.e., b). In particular, the central incisor has a distinctive "square" appearance. b Digital model of the frontal (left) and occlusal (right) views of a healthy control (female).

Discussion

We suggest that quantification of tooth crown shapes may contribute to the early diagnosis of congenital anomaly syndromes involving teeth. A "shape database" that includes associated gene mutations resulting from developmental syndromes may facilitate the genetic identification of hypodontia cases. So far, the authors have not found an existing technique that enables volumetric measurements in isolated teeth for this purpose; only 2D techniques have been described in the literature.

Teeth are multi-dimensional, irregular objects and are, therefore, difficult to measure in 2D. To measure 2D parameters such as the perimeter correctly, the 2D picture must be perpendicular to the occlusal plane. [23] However, each tooth has a specific angulation within the jaw. The analysis of multiple teeth would require a separate picture for each individual tooth. Also, additional phenotypes of tooth morphology and dimensions that involve volumetric aberrations can only be obtained from 3D measurements. Because both mesiodistal and buccolingual dimensions in subjects affected with hypodontia are generally smaller, tooth volume is expected to be an even more discriminative parameter.

Because of scattering and related issues, computed tomography (CT) cannot produce 3D data in sufficient detail to allow detailed geometric measurements of the teeth. Other disadvantages of CT are that it would require exposure of a subject to radiation, and CT equipment is not yet generally available. A laser scanner-based image analysis system that can acquire 3D data of small objects, such as tooth crowns in dental casts, has been described in the literature. The system was considered reliable when comparing the same parameters in 2D and 3D; however, it has not yet been applied to obtain 3D data, such as tooth volume, and would require an experimental setup, which is not very practical. [35]

The drawbacks of other techniques have led to the development of the currently described method of 3D analysis to quantify tooth morphology. The method applied is relatively simple, minimally invasive, and inexpensive compared to other methods and conventional gene tests. The only required input is a dental impression of the upper jaw, which is transferred to a 3D digital model. Direct intra-oral scanning of tooth crowns to obtain a 3D digital dataset, without requiring an impression, is also a realistic option. Presently, the described method is rather time-consuming, although this concern could be resolved considerably by automating the process, which would probably enhance measurement precision as well.

In the present *MSX1* family, a specific pattern of tooth agenesis was observed; however, aberrations in tooth morphology were also noted. The central incisor and first molar were chosen because they present in the mouth at an early age (6 years of age) and because they are almost never congenitally absent, even in cases of severe hypodontia. [3] Future studies may involve other tooth types as well. This may help diagnose other genotype–phenotype correlations. Affected teeth may be different among diseases. To ensure accurate and reproducible measurements, the reference points were chosen on the hard tissues and in non-abrasive zones. Reference points in the vicinity of the gingival

margin were avoided; they were deemed unreliable because of the soft nature of the gingiva and variation due to gingivitis. Furthermore, only the supragingival area of a tooth is available for evaluation, and the size of the area is dependent upon the eruption phase.

Striking features of the central incisor are significantly larger area of the reference plane and a larger buccolingual distance and volume. We did not take the body size into account because this information was not available for the controls. This may have helped in interpreting these findings. *MSX1* cases also present with incisors that have a more square appearance (outcome variable: "squareness"). These findings are in contrast with the finding that all teeth that develop in hypodontia are generally smaller than in control groups. [36, 37]

A possible explanation for the enlarged incisors in the studied family may arise from proximal–distal patterning during tooth development. Tucker showed that the developing oral epithelium can be divided into two domains, one distal and one proximal. [38] The epithelium of the presumptive incisor domain expresses BMP4, which positively regulates the expression of MSX1 and MSX2 in the underlying neural crest-derived mesenchyme. Meanwhile, *Fgf8* is expressed in the epithelial presumptive molar region and regulates the expression of *Barx1*. *Bmp4* and *Fgf8* negatively regulate each other, thereby restricting *Barx1* expression to the presumptive molar region. The boundary between MSX1 and BARX1 demarcates the presumptive incisor- and molar-forming regions. The crown shape could be changed by manipulating the expression of these signaling factors. When beads with noggin protein, which antagonizes *Bmp* signaling, are placed in the distal mesenchyme, and the expression of *Msx1* is lost, a molar tooth is formed in the presumptive incisor region.

Interestingly, in K14-noggin mice, in which overexpression of noggin blocks *Bmp* signaling, the incisors were thick, wide, and blunt-ended. [39] They stated that subtle differences in the level, distribution, and timing of signaling molecules could have morpho-regulatory consequences. [39] Modulation of Bmp4 signaling can transform a conically shaped tooth into a tooth with a more complex morphology. Because MSX1 plays an important role in Bmp signaling, one can hypothesize that the incisors in this family have a more posterior, molar-like appearance as a result of decreased *MSX1* expression.

No statistically significant differences were observed for any of the six parameters in the first molar. Clinically, we did observe a deviating morphology in the cusps. The results

have a tendency towards a smaller volume and more squared appearance. We expect that these small differences in morphology would also be expressed statistically if the case group was larger. In one case, there was a small extra cusp present. The shape of the tooth crown results from morphogenesis of the epithelium during the cap and bell stages of tooth development, through differential growth and folding of the epithelium. [40] The enamel knots express growth stimulatory signals. It has been demonstrated that apoptosis in the enamel knot plays an important role in regulating tooth size and shape, and the expression of *Bmp4* in the enamel knot is associated with apoptosis. [40, 41]

A model has been presented in which the pattern of tooth cusps is regulated by FGF4, which functions as an activator promoting cusp initiation and growth. Inhibitors, such as BMP4, control the distance between the enamel knots and negatively regulate cusp growth. It has also been reported that using z-VAD-fmk treatment to block apoptosis results in morphological anomalies. The morphology of mouse molars was similar to that observed in human macrodontia. [42]

MSX1 and BMP4 are closely associated during tooth development. BMP4 and MSX1 regulate one another in a positive feedback loop [40] Jernvall et al. suggest that MSX1 is needed for *Bmp4* expression, which in turn induces the expression of *p21*, which is responsible for apoptosis. [43] Thus, apoptosis in the enamel knot is necessary for the proper formation of molar teeth, including appropriate shape and size. It is tempting to speculate that MSX1, by inducing BMP4, is involved in this stage of tooth morphogenesis as well. Such speculation could explain the deviating morphology of the molar cusps presented in the studied family. Reduced *Msx1* expression could result in reduced expression of *Bmp4* and *p21*, which in turn would inhibit apoptosis.

Conclusions

We introduced a novel, 3D technique to measure the aspects of tooth morphology to get a better understanding of dental phenotype–genotype associations. This measurement technique may contribute to earlier diagnosis of some multiple-anomaly congenital syndromes involving teeth anomalies. These findings suggest that MSX1 may play a role not only in tooth patterning but also in tooth morphogenesis, as expressed by distinct shape differences, particularly in maxillary incisors, between the *MSX1* family members and controls regarding the parameters of area, buccolingual distance, squareness, and volume.

Acknowledgements

We are grateful to the affected members of the *MSX1* family for their continued participation. Dr. J. Ruijter of the Department of Anatomy and Embryology of the Academic Medical Center, Amsterdam, The Netherlands is gratefully acknowledged for his input and statistical advice. We would also like to thank N. A. Nagel, technical medicine student at the University of Twente, The Netherlands, who wrote part of the measuring program.

References

1. Schalk-van der Weide Y., Bosman F. Tooth size in relatives of individuals with oligodontia. Arch Oral Biol 1996; 41: 469-472.
2. Polder BJ, Van't Hof MA, Van der Linden FP, Kuijpers-Jagtman AM. A meta-analysis of the prevalence of dental agenesis of permanent teeth. Community Dent Oral Epidemiol 2004; 32: 217-226.
3. Creton MA, Cune MS, Verhoeven JW, Meijer GJ. Patterns of missing teeth in a population of oligodontia patients. Int J Prosthodont 2007; 20: 409-413.
4. Nieminen P. Genetic basis of tooth agenesis. J Exp Zool B Mol Dev Evol 2009; 312B: 320-342.
5. Creton M, Cune MS, de Putter C, Ruijter JM, Kuijpers-Jagtman AM. Dentofacial characteristics of patients with hypodontia. Clin Oral Investig 2010; 14: 467-477.
6. Thesleff I. The genetic basis of tooth development and dental defects. Am J Med Genet A 2006; 140: 2530-2535.
7. Bailleul-Forestier I, Molla M, Verloes A, Berdal A. The genetic basis of inherited anomalies of the teeth. Part 1: clinical and molecular aspects of non-syndromic dental disorders. Eur J Med Genet 2008; 51: 273-291.
8. De Coster PJ, Marks LA, Martens LC, Huysseune A. Dental agenesis: genetic and clinical perspectives. J Oral Pathol Med 2009; 38: 1-17.
9. Brook AH. Multilevel complex interactions between genetic, epigenetic and environmental factors in the aetiology of anomalies of dental development. Arch Oral Biol 2009; 54 Suppl 1: S3-S17.
10. Thesleff I, Vaahtokari A, Kettunen P, Aberg T. Epithelial-mesenchymal signaling during tooth development. Connect Tissue Res 1995; 32: 9-15.
11. Kapadia H., Mues GDSR. Genes affecting tooth morphogenesis. Orthod Craniofac Res 2007; 10: 237-244.
12. Matalova E, Fleischmannova J, Sharpe PT, Tucker AS. Tooth agenesis: from molecular genetics to molecular dentistry. J Dent Res 2008; 87: 617-623.
13. Han D, Gong Y, Wu H, Zhang X, Yan M, Wang X, Qu H, Feng H, Song S. Novel EDA mutation resulting in X-linked non-syndromic hypodontia and the pattern of EDA-associated isolated tooth agenesis. Eur J Med Genet 2008; 51: 536-546.
14. Song S, Han D, Qu H, Gong Y, Wu H, Zhang X, Zhong N, Feng H. EDA gene mutations underlie non-syndromic oligodontia. J Dent Res 2009; 88: 126-131.
15. Hardcastle Z, Mo R, Hui CC, Sharpe PT. The Shh signalling pathway in tooth development: defects in Gli2 and Gli3 mutants. Development 1998; 125: 2803-2811.
16. DiBiase AT, Cobourne MT. Beware the solitary maxillary median central incisor. J Orthod 2008; 35: 16-19.
17. Lexner MO, Bardow A, Juncker I, Jensen LG, Almer L, Kreiborg S, Hertz JM. X-linked hypohidrotic ectodermal dysplasia. Genetic and dental findings in 67 Danish patients from 19 families. Clin Genet 2008; 74: 252-259.
18. Lexner MO, Bardow A, Hertz JM, Nielsen LA, Kreiborg S. Anomalies of tooth formation in hypohidrotic ectodermal dysplasia. Int J Paediatr Dent 2007; 17: 10-18.
19. Kimura R, Yamaguchi T, Takeda M, Kondo O, Toma T, Haneji K, Hanihara T, Matsukusa H, Kawamura S, Maki K, Osawa M, Ishida H, Oota H. A common variation in EDAR is a genetic determinant of shovel-shaped incisors. Am J Hum Genet 2009; 85: 528-535.

20. Dong J, Amor D, Aldred MJ, Gu T, Escamilla M, MacDougall M. DLX3 mutation associated with autosomal dominant amelogenesis imperfecta with taurodontism. Am J Med Genet A 2005; 133A: 138-141.
21. Schalk-van der Weide Y., Steen WH, Beemer FA, Bosman F. Reductions in size and left-right asymmetry of teeth in human oligodontia. Arch Oral Biol 1994; 39: 935-939.
22. Brook AH, Elcock C, al-Sharood MH, McKeown HF, Khalaf K, Smith RN. Further studies of a model for the etiology of anomalies of tooth number and size in humans. Connect Tissue Res 2002; 43: 289-295.
23. Brook AH, Griffin RC, Smith RN, Townsend GC, Kaur G, Davis GR, Fearne J. Tooth size patterns in patients with hypodontia and supernumerary teeth. Arch Oral Biol 2009; 54 Suppl 1: S63-S70.
24. Khalaf K, Smith RN, Elcock C, Brook AH. Multiple crown size variables of the upper incisors in patients with supernumerary teeth compared with controls. Arch Oral Biol 2009; 54 Suppl 1: S71-S78.
25. Kantaputra P, Tanpaiboon P, Porntaveetus T, Ohazama A, Sharpe P, Rauch A, Hussadaloy A, Thiel CT. The smallest teeth in the world are caused by mutations in the PCNT gene. Am J Med Genet A 2011; 155: 1398-1403.
26. Schalk-van der Weide Y., Steen WH, Bosman F. Taurodontism and length of teeth in patients with oligodontia. J Oral Rehabil 1993; 20: 401-412.
27. Seow WK, Lai PY. Association of taurodontism with hypodontia: a controlled study. Pediatr Dent 1989; 11: 214-219.
28. Kan WY, Seow WK, Holcombe T. Taurodontism in children with hypodontia and supernumerary teeth: a case control study. Pediatr Dent 2010; 32: 134-140.
29. Bailleul-Forestier I, Berdal A, Vinckier F, de RT, Fryns JP, Verloes A. The genetic basis of inherited anomalies of the teeth. Part 2: syndromes with significant dental involvement. Eur J Med Genet 2008; 51: 383-408.
30. Plyusnin I, Evans AR, Karme A, Gionis A, Jernvall J. Automated 3D phenotype analysis using data mining. PLoS One 2008; 3: e1742.
31. Khalaf K, Elcock C, Smith RN, Brook AH. Fluctuating dental asymmetry of multiple crown variables measured by an image analysis system. Arch Oral Biol 2005; 50: 249-253.
32. van den Boogaard MJ, Dorland M, Beemer FA, van Amstel HK. MSX1 mutation is associated with orofacial clefting and tooth agenesis in humans. Nat Genet 2000; 24: 342-343.
33. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. Lancet 1986; 1: 307-310.
34. British Standards Institution. Precision of test methods 1: Guide for the determination and reproducibility for a standard test method. BSI. BS597, Part 1. 1975. London.
Ref Type: Report
35. Smith R, Zaitoun H, Coxon T, Karmo M, Kaur G, Townsend G, Harris EF, Brook A. Defining new dental phenotypes using 3-D image analysis to enhance discrimination and insights into biological processes. Arch Oral Biol 2009; 54 Suppl 1: S118-S125.
36. Brook AH, Elcock C, al-Sharood MH, McKeown HF, Khalaf K, Smith RN. Further studies of a model for the etiology of anomalies of tooth number and size in humans. Connect Tissue Res 2002; 43: 289-295.
37. McKeown HF, Robinson DL, Elcock C, al-Sharood M, Brook AH. Tooth dimensions in hypodontia patients, their unaffected relatives and a control group measured by a new image analysis system. Eur J Orthod 2002; 24: 131-141.

38. Tucker AS, Matthews KL, Sharpe PT. Transformation of tooth type induced by inhibition of BMP signaling. Science 1998; 282: 1136-1138.

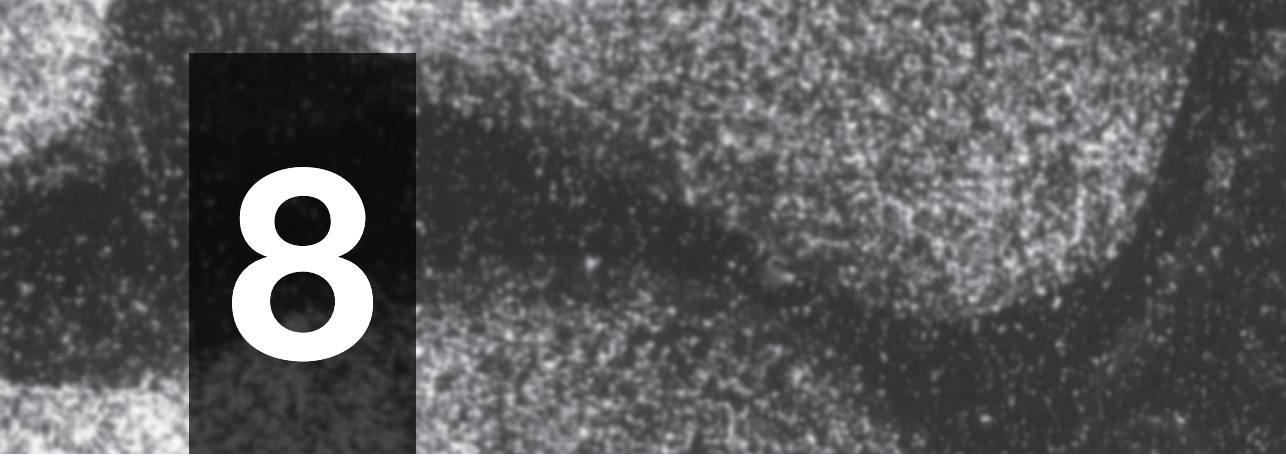
39. Plikus MV, Zeichner-David M, Mayer JA, Reyna J, Bringas P, Thewissen JG, Snead ML, Chai Y, Chuong CM. Morphoregulation of teeth: modulating the number, size, shape and differentiation by tuning Bmp activity. Evol Dev 2005; 7: 440-457.

40. Bei M, Maas R. FGFs and BMP4 induce both Msx1-independent and Msx1-dependent signaling pathways in early tooth development. Development 1998; 125: 4325-4333.

41. Jernvall J, Thesleff I. Reiterative signaling and patterning during mammalian tooth morphogenesis. Mech Dev 2000; 92: 19-29.

42. Kim JY, Cha YG, Cho SW, Kim EJ, Lee MJ, Lee JM, Cai J, Ohshima H, Jung HS. Inhibition of apoptosis in early tooth development alters tooth shape and size. J Dent Res 2006; 85: 530-535.

43. Jernvall J, Aberg T, Kettunen P, Keranen S, Thesleff I. The life history of an embryonic signaling center: BMP-4 induces p21 and is associated with apoptosis in the mouse tooth enamel knot. Development 1998; 125: 161-169.



8

Chapter 8

MSX1 and hydrocephaly

Identification of a missense mutation in the MSX1 homeodomain, in a patient with hydrocephaly.

Van den Boogaard MJ, Gooskens RH, Créton M, Nievelstein RA, Ploos van Amstel HK, Lindhout D

Submitted: Eur J Hum Genet

Abstract

There is increasing evidence that genetic factors play a significant role in the pathogenesis of hydrocephaly. However, to date in humans only one single gene (*L1CAM*) for isolated (non-syndromic) hydrocephaly with aqueduct stenosis has been identified.
In mice, one of the genes associated with hydrocephaly due to aqueduct stenosis is *Msx1*. This prompted us to perform molecular diagnostic evaluation of the *MSX1* gene in patients with hydrocephaly based on aqueduct stenosis without striking additional abnormalities. One proband out of nine unrelated patients examined, indeed showed a novel c.581 A>G (p.Lys194Arg) missense mutation. The transition caused a substitution of a highly conserved amino acid located in the homeodomain of *MSX1*.
Although not conclusive, the above observation is suggestive that in rare cases abnormalities of the *MSX1* gene play a role in the pathogenesis of hydrocephaly due to aqueduct stenosis.

Introduction

Congenital hydrocephalus, characterized by an abnormal accumulation of cerebrospinal fluid (CSF) in the head, has a birth prevalence of 2.2 to 4.8 per 10.000 births.[1] This severe medical condition consists of heterogeneous complex and multifactorial disorders.[2] Non communicating hydrocephalus results from an obstruction of CSF outflow of the ventricular system. This obstruction usually occurs at the level of the cerebral aqueduct. There are many causes for this type of obstructive hydrocephaly including viral infections, tumors, hemorrhage and developmental defects. [2, 3, 4]

There is increasing evidence that also genetic factors play a significant role in the pathogenesis of hydrocephaly. [2, 4, 5]
Familial forms of congenital hydrocephalus, with different modes of inheritance have been reported. [2] However, to date in human patients only one single gene (*L1CAM*) for isolated (non-syndromic) hydrocephaly with aqueduct stenosis has been identified.

Expression studies showed that *Msx1* is one of the genes important during development of the nervous system, and craniofacial, dental and limb formation. [6] As from early stages of development, *Msx1* is expressed in the developing dorsal neural tube and neural crest cells.[7] In later stages, striking expression was observed in the circumventricular organs as are the subcommissural organ (SCO) and choroid plexus, and in epithelia of the dorsal parts of the third ventricle. [8]

Studies showed that most but not all *Msx-/-* mutants, besides the craniofacial defect and tooth agenesis, have aqueductal collapse and severe hydrocephalus. [9, 10, 11,12, 13]
These manifestations coincided with a reduction in size of the cerebral cortex, the caudate putamen, the septum and the corpus callosum. [12, 13] An important feature observed in *Msx1*-deficient mice was also absence or malformation of the posterior commissure and subcommissural organ (SCO). [13] A normal development and functioning of the SCO is of great importance for the maintenance of an open aqueduct and for the normal circulation of the cerebrospinal fluid. [4, 14, 15, 16, 17] (Figure 8.1)

Figure 8.1

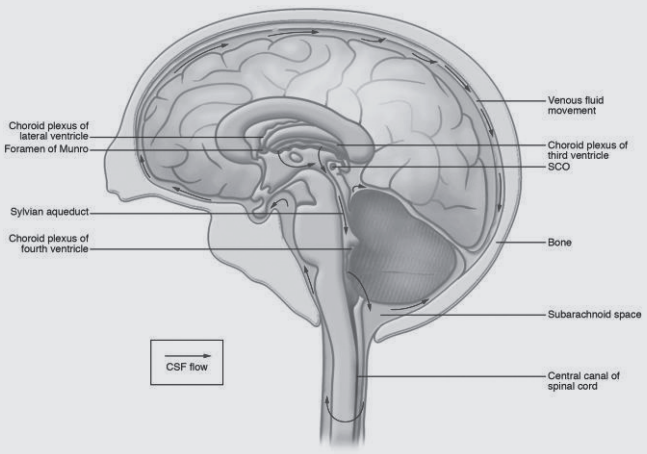


Figure 8.1 CSF flow and the SCO | CSF is secreted from the choroid plexus and travels through the ventricular system in a rostrocaudal direction from the lateral ventricles to the third ventricle via the foramen of Munro, then through the Sylvian aqueduct to the fourth ventricle, and finally into the cisterna magna of the subarachnoid space and the central canal of the spinal cord. CSF fluid is removed through the arachnoid villi into the venous circulation. Adapted from Picketts et al. (2006) – by permission from American Society for Clinical Investigation.

Recently, a reduced expression of *Msx1* was demonstrated in a *Sox3* transgenic mice showing congenital non-communicating hydrocephaly with abnormal SCO development. [18]
These studies implicate the involvement of *Msx1* in the etiology of aqueduct stenosis resulting in hydrocephaly. [13]
Interestingly, histological studies showed that also in man a SCO is formed. The human SCO reaches its full development during embryonic life and undergoes regressive changes after birth. [15] It was demonstrated that human hydrocephalic fetuses presented a size reduction of the subcommisural organ. [19]

Although the role of *MSX1* in the etiology of hydrocephaly in human is currently unknown, the above observations prompted us to perform molecular genetic analysis of the *MSX1* gene in a diagnostic setting of a small series of patients with hydrocephaly due to aqueduct stenosis of unknown cause.

Methods

Cases

Background population: In total 123 individuals with hydrocephaly were known at the department of pediatric neurology of the University Medical Center Utrecht (UMCU).
In total 27 patients were classified as hydrocephalus due to aqueduct stenosis without a specific diagnosis. In 18 patients with hydrocephaly due to aquaduct stenosis visiting the department of pediatric neurology, diagnostic DNA analysis was performed at the department of Medical Genetics of the UMCU. In 9 patients fulfilling the criteria: i) hydrocephaly due to aqueduct stenosis without a (suggestive) diagnosis; ii) a *L1CAM* mutation excluded; iii) normal karyotype in the past, additional analysis of *MSX1* was performed.

Family members of the single patient with a *MSX1* mutation were asked for cooperation according to standard clinical genetics protocols including phenotypic evaluation, molecular genetic testing and, if appropriate and requested, genetic counseling. When a family member was tested positive for the mutation, phenotypic analysis was extended by MRI of the brain and dental examination by a dentist.

Mutation analysis of *MSX1*

High molecular weight genomic DNA was extracted from blood samples using standard procedures. For mutation analysis of the coding region of MSX1 the exons 1 and 2 were amplified using PCR according to established procedures. Exon 1 was amplified in two overlapping fragments with primers sets
MSX1-E01-01F(5'CCCGGAGCCCATGCCCGGCGGCTG3') and
MSX1-E0101R(5'CTAGCACCGGCTGCAGGTACGCA3'),
MSX1-E01-02F(5'AAAGTGTCCCCTTCGCTCCTGCCC3') and
MSX1-E01-02R (5'CTAGCACCGGCTGCAGGTACGCA 3').
The two primer sets for exon 2 are MSX1-E02-01F
(5'CTATTATTACTACTTCTTGGGCTGATCAT3') and
MSX1-E02-01R (5'CTCTGCCTCTTGTAGTCTCTTTG3'),
MSX1 E02-02F (5' CACTGAGACGCAGGTGA3') and
MSX1 E02-02R (5'CTGGAGGAATCGGCTGG3').

Sequencing was performed with the ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction KIT (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Sequencing products were analyzed using an ABI 3730/3730xl DNA Analyzer (Applied Biosystems). Nucleotide numbering of the mutations is according to the cDNA numbering with 1 corresponding to the A of the ATG translation initiation codon in the MSX1 reference sequence (NM_002448.3). To predict the biological relevance of variants on protein function we used the mutation interpretation software package Alamut 2.0 (Interactive Biosoftware, Rouen, France).

Results

Mutation analysis

Mutation analysis of the *MSX1* gene revealed heterozygosity for a novel c.581 A>G (p.Lys194Arg) missense mutation in a single patient (III3, figure 8.2).
The c.581 A>G (p.Lys194Arg) mutation is encoded by exon 2 and located in the homeodomain. Indicators of pathogenicity were not unambiguously conclusive with a Grantham Distance of 25, an Align-GVGD class 25, SIFT as not tolerated and a Polyphen-2 score of 0.965 (sensitivity 0.77, specificity 0.95). Subsequently, segregation analysis was performed in the family to establish affection status among family members.

The mutation was present in the index case (III3), his mother (II2), one aunt (II6) and two uncles (II5; II7) (Figure 8.2). This mutation was not found in a panel of 100 normal controls chromosomes. However, recently in a panel of 10.000 exomes (Exome Variant Server, NHLBI Exome Sequencing Project (ESP), Seattle, WA (URL: http://evs.gs.washington.edu/EVS/) [July 2012 accessed] this mutation has been found in 3 out of 13.001 alleles tested.

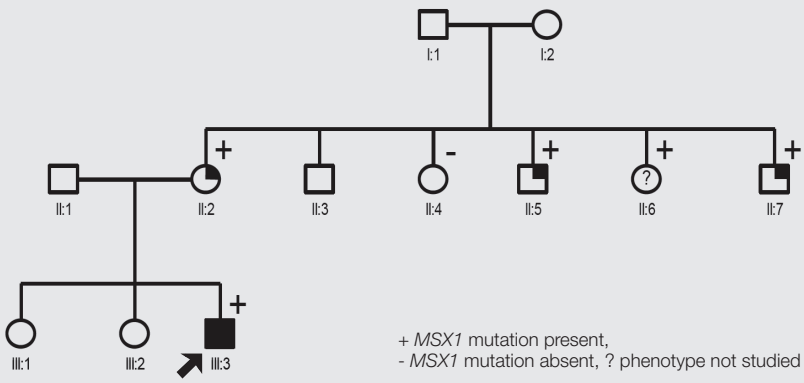


Figure 8.2

Figure 8.2 Pedigree of patient with hydrocephaly and *MSX1* mutation.

Clinical characteristics *MSX1* proband

The identified *MSX1* mutation carrier (III3, Figure 8.2) was a 16-year old boy, with congenital hydrocephalus based on an aqueduct stenosis. He is the third child to a healthy non-consanguineous Caucasian couple. He was born at term, after an uneventful pregnancy. At three weeks of age, an increasing head circumference was noted. MRI revealed an aqueduct stenosis. Ventriculo-peritoneal drainage was performed at the age of four weeks. On physical examination at the age of 16 years no additional anomalies and/or dysmorphic features were present. His growth parameters were normal.

MRI following shunting for hydrocephaly demonstrated a somewhat irregularly shaped ventricular system, with more expansion of the right lateral ventricle than the left, and a deformed corpus callosum, with thinning of the truncus and splenium. The amount of white matter was reduced (Figure 8.3 A,B).

Dental investigation, including panoramic radiographs showed no abnormalities.

Figure 8.3

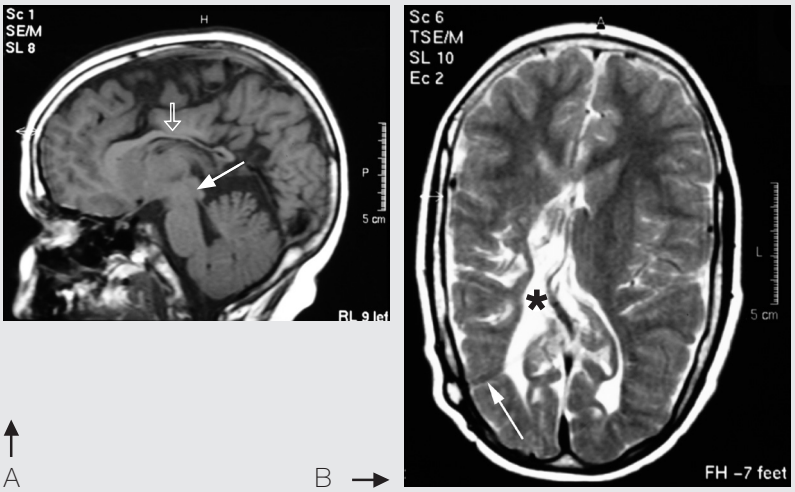


Figure 8.3 MRI of the brain in proband III3, figure 8.2. A. Midsagittal T1 Spin Echo (SE) image illustrating the irregular thinning of the corpus callosum (open arrow) as well as the obliteration of the lumen of the aqueduct (arrow). B. Axial T2 SE image after ventriculo-peritoneal drainage illustrating the somewhat irregular shaped ventricular system, with more expansion of the right lateral ventricle than the left (asterisk), and a diminished amount of white matter. The VP drain is present in the right occipital horn of the lateral ventricle (arrow).

Clinical characteristics in *MSX1* family members

To establish the phenotypic expression of the c.581 A>G (p.Lys194Arg) *MSX1* mutation and to identify dental features of *MSX1*, family members carrying this mutation, were examined by a dentist. Panoramic dental radiographs were made.

Absence of several teeth was observed in the mother and uncles of the proband. Unfortunately, no medical data were available.

The mother of patient 1 (II2, Figure 8.2) missed two premolars (15 en 45). Her teeth were normal in size and morphology. She had no recall of extraction of permanent teeth in childhood, and therefore we presumed that these premolars were agenetic, rather than having been extracted.

In an uncle of patient 1 (II5, Figure 8.2) absence of several teeth was observed, including absence of canines. One canine (13) showed an abnormal morphology, resembling both a canine and premolar. The patient recalled several extractions over the years. Therefore,

we were not able to determine which teeth had been extracted and which teeth, if any, were congenitally absent.
The second uncle (II7, figure 8.2) showed absence of two premolars (15, 25) and two third molars (38, 48). No abnormalities in size and morphology were observed.
MRI of the brain in the mother of patient 1 showed no abnormalities. The MRI of the brain of the uncle showed a slight irregular thinning of the truncus of the corpus callosum with an open aqueduct (Figure 8.4). Flow measurements through the aqueduct did reveal diminished flow in the aqueduct.

Figure 8.4

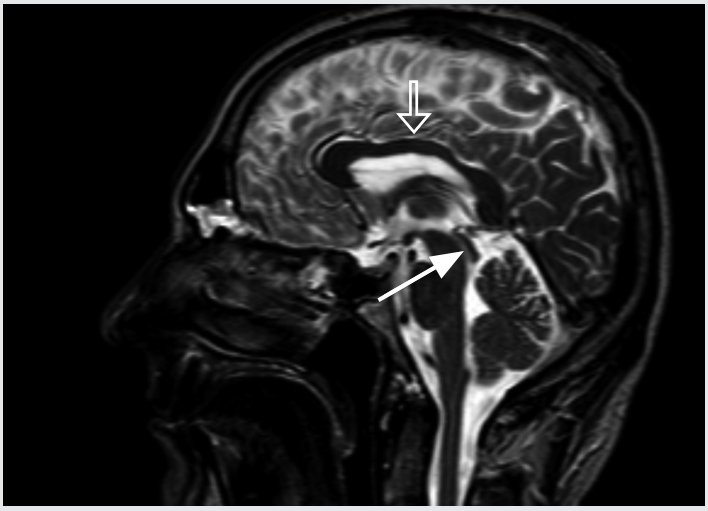


Figure 8.4 MRI of the brain in the uncle of th proband (II7, figure 8.2). The midsagittal T2 Turbo Spin Echo (TSE) image shows the slightly irregular thinning of the truncus of the corpus callosum (open arrow). An aqueductal flow void is lacking (arrow). Flow measurements through the aqueduct did reveal diminished flow through the aqueduct (not shown).

Discussion

In this paper we present a patient with hydrocephaly with aqueduct stenosis carrying a *MSX 1* mutation c.581 A>G (p.Lys194Arg) located in the highly conserved homeodomain of the protein. The mutation is located at the end of the first helix of the homeodomain.

This first helix is important for stabilization of the protein, for transcriptional repression and for protein-protein interaction. [20] It was demonstrated that *Msx1*-deficient mice exhibited hydrocephaly with absence or malformation of the posterior commissure and the subcommissural organ with collaps of the cerebral aqueduct. The role of *MSX1* in the aetiology of aqueduct stenosis in human patients remains to be elucidated.

The MRI of the c.581 A>G (p.Lys194Arg) carrier with congenital hydrocephaly due to aqueduct stenosis showed an irregular ventricular system and a deformed corpus callosum with thinning of the truncus and splenium. A reduction in size of the corpus callosum was also detected in the brain of *Msx1* mice mutants. [12] However, morphostructural anomalies of the corpus callosum have been described in subjects with hydrocephaly, independently of the etiology. [12] Furthermore, MRI of patients with hydrocephaly, prior to or following shunting can also show corpus callosal changes. [21, 22] Nevertheless, it is remarkable that the MRI of the uncle of our patient with hydrocephaly, carrying the *MSX1* mutation, reveals changes of the corpus callosum without hydrocephaly but with slowing of aquaductal flow. This suggests that these corpus callosum abnormalities and functional changes of the aqueduct may be features caused or influenced by the *MSX1* mutation. However, the role of *MSX1* in this family is not indisputable because the brain MRI in the mother of the patient, also carrying the *MSX1* mutation, showed no abnormalities. Furthermore, one has to keep in mind that interpretation software suggests pathogenicity of the mutation albeit not unequivocally: the mutation is considered as an unclassified variant.

The absence of hydrocephaly in carriers of the novel *MSX1* mutation might also be explained by a variable degree of expression or penetrance as observed in mice: not all *Msx1* mouse mutants exhibited severe hydrocephaly and aqueduct stenosis. [12, 13]

Picketts (2006) support the view that hydrocephaly is an oligogenic trait and stated that a combination of a reduced cell number within the SCO, a disrupted function of the SCO and an abnormal cilia function of the ventricular ependymal cells lining the cerebral aqueduct significantly may alter the laminar flow and could initiate or promote the development of hydrocephaly. [4] One can hypothesize that the hydrocephaly in our patient is caused by the simultaneous presence of mutations in different genes, one of which is the *MSX1* mutation and also involves the above postulated mechanisms.

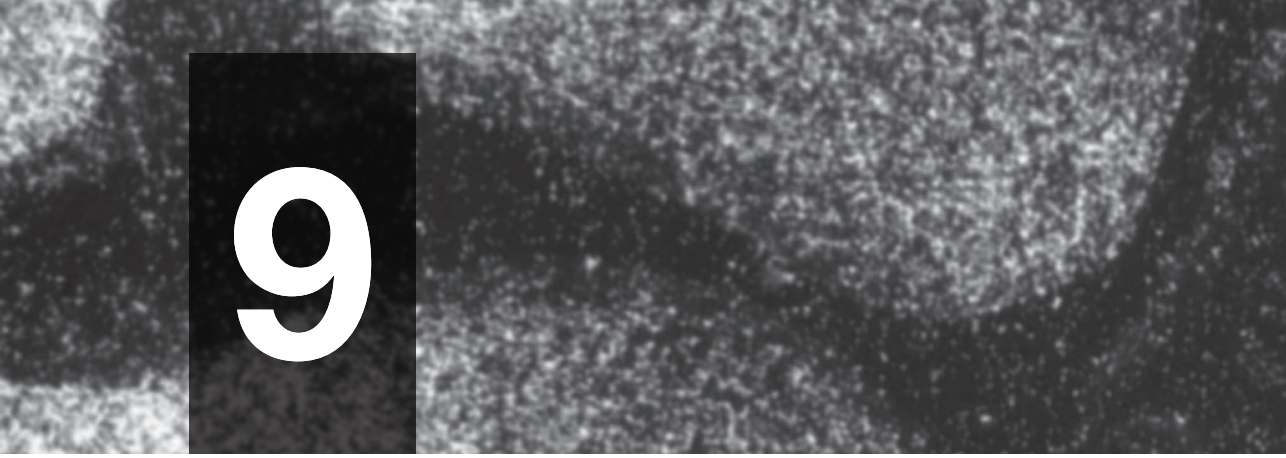
Although *MSX1* is essential in tooth development [23] , the clinical data suggest that the identified mutation has not had a major effect on tooth development, although in both the mother and uncle premolars were absent. Premolars are often absent in *MSX1* families with tooth agenesis. [24]

In summary, we present a novel *MSX1* mutation in a patient with aqueduct stenosis. We propose that the *MSX1* gene might be a candidate for hydrocephaly in humans albeit in rare cases. Genetic modifiers like additional mutations in other candidate genes might determine the presence and severity of symptoms, or, alternatively, *MSX1* may be a genetic modifier for other genes related to hydrocephaly due to aquaductstenosis. We realize that we studied just a small number of patients and the presence of *MSX1* mutation and aqueduct stenosis is reported for only a single case. A search for mutations in *MSX1* for aqueduct stenosis in a larger panel of patients with non-syndromic hydrocephaly is warranted to further explore the possible role of *MSX1* in hydrocephalus due to aqueduct stenosis in human patients.

References

1. Garne E, Loane M, Addor MC, Boyd PA, Barisic I, Dolk H. Congenital hydrocephalus--prevalence, prenatal diagnosis and outcome of pregnancy in four European regions. Eur J Paediatr Neurol. 2010 Mar;14(2):150-5.
2. Zhang J, Williams MA, Rigamonti D. Genetics of human hydrocephalus. J Neurol. 2006 Oct;253(10):1255-66.
3. Cinalli G, Spennato P, Nastro A, Aliberti F, Trischitta V, Ruggiero C, Mirone G, Cianciulli E. Hydrocephalus in aqueductal stenosis. Childs Nerv Syst. 2011 Oct;27(10):1621-42.
4. Picketts D.J. Neuropeptide signaling and hydrocephalus: SCO with the flow The J. of Clin Invest. 2006; 116:1828-1832
5. Verhagen JM, Schrander-Stumpel CT, Krapels IP, de Die-Smulders CE, van Lint FH, Willekes C, Weber JW, Gavilanes AW, Macville MV, Stegmann AP, Engelen JJ, Bakker J, Vos YJ, Frints SG. Congenital hydrocephalus in clinical practice: a genetic diagnostic approach. Eur J Med Genet. 2011 Nov-Dec;54(6):e542-7.
6. Bendall AJ, Abate-Shen C. 2000. Roles for Msx and Dlx homeoproteins in vertebrate development. Gene. 2000 Apr 18;247(1-2):17-31.
7. Bach A, Lallemand Y, Nicola MA, Ramos C, Mathis L, Maufras M, Robert B. Msx1 is required for dorsal diencephalon patterning. Development. 2003 Sep;130(17):4025-36.
8. Ramos C, Martinez A, Robert B, Soriano E. Msx1 expression in the adult mouse brain: characterization of populations of beta-galactosidase-positive cells in the hippocampus and fimbria. Neuroscience. 2004a;127(4):893-900.
9. Satokata I, Maas R Msx1 deficient mice exhibit cleft palate and abnormalities of craniofacial and tooth development.Nat Genet. 1994;6:348-56.
10. Houzelstein D, Cohen A, Buckingham ME, Robert B. 1997. Insertional mutation of the mouse Msx1 homeobox gene by an nlacZ reporter gene.
Mech Dev. 1997 Jul;65(1-2):123-33.
11. Jumlongras D, Bei M, Stimson JM, Wang WF, DePalma SR, Seidman CE, Felbor U, Maas R, Seidman JG, Olsen BR. 2001. A nonsense mutation in MSX1 causes Witkop syndrome.Am J Hum Genet. 2001 Jul;69(1):67-74
12. Ramos C, Fernández-Llebrez P., Bach A, Robert B, Soriano E. MSX1 disruption leads to diencephalons defects and hydrocephalus Dev Dyn 2004;230:446
13. Fernández-Llebrez P, Grondona JM, Pérez J, López-Aranda MF, Estivill-Torrús G, Llebrez-Zayas PF, Soriano E, Ramos C, Lallemand Y, Bach A, Robert B. Msx1-deficient mice fail to form prosomere 1 derivatives, subcommissural organ, and posterior commissure and develop hydrocephalus. J Neuropathol Exp Neurol. 2004 Jun;63(6):574-86.
14. Meiniel A. The secretory ependymal cells of the subcommissural organ: Which role in hydrocephalus? The Int. J of Biochem and Cell Biol 2007;39:463-468
15. Galarza M. Evidence of the subcommissural organ in humans and its association with hydrocephalus Neurosurg Rev 2002;25:205-215
16. Pérez-Fígares JM, Jimenez A J, Rodrígues E M. Subcommissural organ, cerebrospinal fluid circulation, and hydrocephalus. Micr.Res and Technique 2001; 52:591-607
17. Huh MS, Todd MA, Picketts DJ. SCO-ping out the mechanisms underlying the etiology of hydrocephalus.Physiology (Bethesda). 2009 Apr;24:117-26. Review.

18. Lee K, Tan J, Morris MB, Rizzoti K, Hughes J, Cheah PS, Felquer F, Liu X, Piltz S, Lovell-Badge R, Thomas PQ. Congenital hydrocephalus and abnormal subcommissural organ development in Sox3 transgenic mice. PLoS One. 2012;7(1):e29041.
19. Castañeyra-Perdomo A, Meyer G, Carmona-Calero E, Bañuelos-Pineda J, Méndez-Medina R, Ormazabal-Ramos C, Ferres-Torres R. Alterations of the subcommissural organ in the hydrocephalic human fetal brain. Brain Res Dev Brain Res. 1994 Jun 17;79(2):316-20.
20. Hu G, Vastardis H, Bendall AJ, Wang Z, Logan M, Zhang H, Nelson C, Stein S, Greenfield N, Seidman CE, Seidman JG, Abate-Shen C.Mol Cell Biol. 1998 Oct;18(10):6044-51.
21. Constantinescu C S, Mc conachie N S, White B D. 2005 Corpus callosum changes following shunting for hydroceohalus: case report and review of the literature.
22. Lane J I, Luetmer P H, Atkinson J L. (2001) Corpus callosal signal changes in patients with obstructive hydrocephalus after ventriculoperitoneal shunting. AJNR Am J Neuroradiol. 22:158-162
23. Nieminen P. 2009 Genetic basis of tooth agenesis. J Exp Zool B Mol Dev Evol. Jun 15;312B(4):320-42.
24. Kim JW, Simmer JP, Lin BP, Hu JC (2006). Novel MSX1 frameshift causes autosomal-dominant oligodontia. J Dent Res 85:267-271



9

Chapter 9

General Discussion and Summary

Future Prospects

Nederlanse samenvatting (Dutch Summary)

Curriculum Vitae

Dankwoord (Acknowledgements)

List of publications

Addenda

General Discussion and Summary

Linking clinical, developmental and molecular approach in clinical practice

A woman with hypodontia was referred to our clinic. Her brother is born with a cleft palate and also misses several teeth. He is mentally retarded. "

Orofacial clefting and hypodontia are both common congenital disorders. [1-4]
In genetic counseling, it is often difficult to identify the underlying cause of both disorders. Both have a complex etiology, in which genetic and environmental factors might play a role. [1, 2, 4] The recurrence risk of orofacial clefting is often based on empirical data. [4]
A number of studies demonstrated a role for genetic factors in the etiology of tooth agenesis. [6] Patients with hypodontia frequently have an affected family member. [6, 7, 8, 9] However, only in the minority of the patients a single genetic factor could be identified. [9]

In this thesis we described the molecular pathogenesis of clefting and hypodontia, and more specifically studied the role of *MSX1* in the etiology of both disorders in the Dutch population.
In addition, we evaluated hydrocephaly as a possible feature of the phenotypic spectrum of *MSX1*. Furthermore, we assessed the contribution of *WNT10A* in the etiology of isolated hypodontia.

Clefting and hypodontia; clinical approach

In the family presented in **chapter 3** and in the above-introduced family orofocial clefting as well as hypodontia segregated. In some family members both anomalies were present. Since orofacial clefting and hypodontia are both common disorders, one has to take coincidence into account. On the other hand, the presence of both malformations might well be related.
Orofacial clefting is often associated with additional anomalies, including major and minor defects. Based on the systematic review we performed **(chapter 2)**, the prevalence of associated anomalies in cleft lip (CL) and lip palate (CLP)/ cleft lip with or without cleft palate (CL±P), was respectively ~10% and ~25%. CP was most frequently associated with additional anomalies (45.9%; range 22.2–78.3%). [10]

A number of studies reported a significant association between orofacial clefting and dental anomalies. [11, 12, 13] Recently, it was demonstrated that dental anomalies, including tooth agenesis, are commonly found in unaffected siblings and parents of children born with clefts in comparison with families with no family history of clefts. [14] These studies suggest the involvement of shared genetic factors responsible for the presence of clefting and tooth agenesis in these families.
In **chapter 1** we give more insight in the related etiology of orofacial clefting and hypodontia based on embryology, molecular mechanisms and a selection of syndromes associated with both congenital anomalies, based on the currently available knowledge.

From a clinical perspective, when a family with orofacial clefting and/or hypodontia is referred for genetic counseling, at first the possibility of a specific syndrome diagnosis or chromosomal anomaly should be taken into consideration. To date, approximately 50 syndromes are associated with both clefting and hypodontia (OMIM website 15; London Medical Databases (LMD) [16]; **chapter 1**). While, the presence and type of associated anomalies can be indicative for a specific diagnosis, we advise physical examination, including an accurate dysmorphologic and dental examination, in patients with orofacial clefting and/or tooth agenesis. Also follow-up of patients is important to detect possible features developing later in life. In addition, a structured interview focusing on symptoms of a syndrome diagnosis or chromosomal anomaly is of importance, as well as a structured and comprehensive family history, including cancer (indicative for a mutation in e.g. *PTCH1* (**chapter 1**) and *AXIN2* (**chapter 6**)), should be obtained. In some patients physical examination of family members may be indicative for a syndrome diagnosis or for a single gene that might be involved. However, in the absence of a specific phenotype and of clues for a syndrome diagnosis, monogenetic disorder or chromosomal anomaly another approach with a broader view, including mouse models (**chapter 3**) might be required.

Clefting and hypodontia; integrated approach and gene identification

In **chapter 3,** we investigated a family with orofacial clefting in combination with hypodontia. Clinical investigation did not lead to a specific syndrome diagnosis.
Interestingly, a combination of cleft palate and tooth agenesis was previously reported in the *Msx*-deficient mouse. [17] Expression studies in mice have demonstrated a striking expression of this gene in the facial processes and teeth. [18, 19] In addition, association

studies suggested *MSX1* as a candidate gene for orofacial clefting in humans. [20] However, subsequent sequencing studies in patients with orofacial clefting did not reveal a pathogenic mutation supporting this hypothesis. [20]
Based on the above, we defined *MSX1* as a suitable candidate gene for orofacial clefting in combination with hypodontia and sequenced *MSX1* in the affected family members. With this candidate gene approach we could identify *MSX1* as a cause of autosomal dominant orofacial clefting with tooth agenesis. [21] Moreover, we confirmed *MSX1* is an interesting candidate gene for orofacial clefting in human.

After we established *MSX1* as a candidate gene involved in both cleft palate and tooth agenesis in humans, complete sequencing of *MSX1* in a large population by Jezewski et al. [22] revealed that 2% of the patients with cleft lip and/or palate carry a mutation in *MSX1.* Additional association studies confirmed the role of *MSX1* in the etiology of non-syndromic clefting in different populations. [23-35] Recently, a novel *MSX1* nonsense mutation causing hypodontia and clefting was identified. [36]

Interestingly, results of association studies were suggestive for an interaction between *MSX1* and *IRF6*, involved in the etiology of cleft lip with or without cleft palate [35] and tooth agenesis. [37] As mentioned (Chapter 1), *IRF6* is responsible for Van der Woude syndrome (OMIM 119300), in which orofacial clefting and also tooth agenesis are significant features. Murray and Schutte [38] noticed that mutations in *IRF6*, *MSX1*, as well as *FGFR1* are associated with dental anomalies and different types of orofacial clefting. Based on this, the categories of orofacial clefting that are presented in families might give a clue for the candidate gene involved in the etiology of these clefts.

In **chapter 4**, we describe the developmental and molecular pathogenesis of *MSX1* mutations in relation to orofacial clefting and tooth agenesis based on literature.
Expression studies demonstrated that *Msx1* is broadly expressed in a variety of tissues during embryonic development and important in both craniofacial and dental development. Striking expression is seen in zones where ectodermal-mesodermal interaction takes place. [39] MSX1 has a main role in crucial processes in morphogenesis, including proliferation and differentiation important for morphogenesis. [39] *Msx1* mouse mutants manifest multiple craniofacial defects, including complete cleft palate, deficient development of the alveolar bone of the mandible and premaxilla, abnormalities of the malleus in the middle ear, and complete tooth agenesis. [17]
In *Msx1* mouse mutants a decrease in cell proliferation in palatal and dental mesenchyme

was observed. [40, 41] This observation suggests that orofacial clefting in *Msx1* mutations is mainly caused by a disruption in growth. [40]
Furthermore, MSX1 is essential for the establishment of the odontogenic potential of the mesenchyme through the maintenance of mesenchymal *Bmp4* expression. [42] It is demonstrated that the absence of BMP4 in dental mesenchyme in the *Msx1* mutant account for the arrest in tooth development at the bud stage. [43] The deficient development of the alveolar bone suggests a differentiation defect due to down-regulation of *Bmp4* expression. [44, 45]

Addition of BMP4 to *Msx1*- deficient mice could rescue the palate phenotype and partly the arrest in tooth development. [40] Another study demonstrated that overexpression of *Bmp4* as well as modulation of Shh signaling by inhibition of *Dlx5* can rescue cleft palate in *Msx1* knockout mice. [46]
The above- discussed studies demonstrate the significance of *Bmp4* as a downstream target of *Msx1,* in palate and dental development and in alveolar bone formation. [41, 44-47]
Recently, *BMP4* analysis in patients with orofacial clefting revealed mutations in *BMP4* in patients with different cleft categories, similar to *MSX1*. [47] Interestingly, these cleft categories included cleft lip and palate, microform of cleft lip in combination with cleft palate, and a microform cleft lip with uvula bifida and subepithelial defects. Different cleft categories were also present within the same family. The overrepresentation of *BMP4* mutations in microform cleft lip supports that *BMP4* may underlie a subset of orofacial clefting in humans. [47]

We considered (**chapter 4**) the different cleft categories and the tooth agenesis present in the *MSX1* family and the cleft categories reported in *BMP4* families in line with the expression pattern and role of *MSX1* in proliferation and differentiation during embryonic development.

Our finding of the *MSX1* nonsense mutation being responsible for autosomal dominant clefting in combination with hypodontia presented in **chapter 3** and the reported *BMP4* mutations in patients with orofacial clefting demonstrate the benefit of integrating embryology, mouse models and molecular pathways in the identification of a candidate gene in clinical practice. This approach could also be considered in other patients referred for syndrome diagnostics and genetic counseling.
The benefit of integrating syndromology and molecular pathways can be illustrated by our finding that *WNT10A* often contributes to the etiology of isolated tooth agenesis. (**chapter 6**).

We already mentioned that Wnt/ β-catenin signaling pathway regulates many processes in development (**chapter 1**). Several studies suggest that *Wnt10* and *Msx1* are involved in the same pathway during tooth development. *Msx1* expression is under control of WNT-induced expression of *FgF4* and *Fgf9*.[48] It was demonstrated that *Wnt10A* is expressed during different stages of tooth development, which support a role of *Wnt10A* in tooth morphogenesis and differentiation of odontoblasts. [49] In humans, *WNT10A* mutations were associated with Odontoonychodermal dysplasia (OODD OMIM 257980) characterized by severe hypodontia, nail dystrophy, palmoplantar keratoderma, hyperhidrosis and hypotrichosis. [50] Interestingly, Bohring et al. found that heterozygous carriers of a *WNT10A* mutation might show mild features, including mainly tooth and nail anomalies. [51]

Based on the above; we defined *WNT10A* as a good candidate gene for isolated tooth agenesis and included *WNT10A* in the genetic evaluation of a panel of patients with hypodontia (**chapter 6**). In parallel with our study, a family with isolated hypodontia with two *WNT10A* missense mutations was reported, further supporting *WNT10A* as an interesting candidate gene. [52] Our study revealed that the rate of *WNT 10A* mutations in patients with non-syndromic hypodontia is surprisingly high (**chapter 6**). [8]

MSX1 in relation to non-syndromic clefting

In **chapter 5 and 6** our aim was to gain more insight in the contribution of *MSX1* in non-syndromic orofacial clefting and hypodontia in the Dutch population.

The presented *MSX1* mutation, causative for clefting in combination with hypodontia, was identified in a Dutch family. We have already mentioned that several studies confirmed the role of *MSX1* in the etiology of non-syndromic clefting in different populations. [23-35]

To assess the role of *MSX1* in the etiology of non-syndromic orofacial clefting in the Dutch population, we performed a case-control triad (mother, father, and child) study. There was increasing evidence that gene-environment interaction play a role in determining susceptibility to orofacial clefting. [23, 38, 53, 54] Furthermore, previous studies support an interaction between environmental factors and *MSX1.* [23, 55, 56] Therefore, we investigated the interactions between *MSX1* and the parents' periconceptional lifestyle in relation to the risk of OFC in their offspring (**chapter 5**). [57]

In this study, we could not confirm a significant association between orofacial clefting and

MSX1 in the Dutch population. However, the results supported an interaction between lifestyle factors and *MSX1*. Our data suggests that periconceptional smoking by both parents, but particularly by the mother, interacts with a specific allelic variant of *MSX1* to increase risk of orofacial clefting in their offspring. Because of its relevance in periconceptional counseling further studies with a large sample size, particularly studying both parents' smoking, is warranted.

We realize that in case-control studies recall and reporting bias must be considered. Because of guilt-feelings, case mother might have underreported their smoking habits. However, studies reported that the validity of reporting smoking was rather excellent. Although, this was somewhat less for the level of smoking reported. [58] One might hypothesize that when both parents smoked, the number of cigarettes smoked might be larger. However the increased orofacial cleft risk when both parents smoked could reflect an biparental additive dose-response effect. A (mild) dose-reponse effect for smoking and orofacial cleft risk has been reported in previous studies. [59, 60, 61, 62]

In **chapter 5** we postulated that the increased risk between smoking and *MSX1* might be explained by several mechanisms, including alteration in expression of genes involved in similar cellular processes as *MSX1* (cell cycle/ proliferation/differentiation) and gene-gene interaction. Further studies are needed to evaluate these possible mechanisms, and to draw causal inferences.
In conclusion, based on our study we conclude that the contribution of *MSX1* in the etiology of orofacial clefting in the Dutch population is small, and that a possible interaction between smoking and *MSX1* might increase orofacial cleft risk.

MSX1 in relation to non-syndromic hypodontia

We already discussed *Msx1* is significant in tooth development and that absence of MSX1 in mice results in a complete tooth agenesis with an arrest of development in the bud stage (**chapter 1**). [17]

To further investigate the role of MSX1 in the etiology of tooth agenesis in the Dutch population, we studied a panel of patients with hypodontia (**chapter 6**). [8] In this study we performed sequence analysis of *MSX1* in a panel of hypodontia patients, classified as non-syndromic and syndromic hypodontia. In addition, we assessed the mutation frequency of four additional candidate genes (*PAX9*, *IRF6*, *AXIN2* and *WNT10A)*.

This analysis revealed that *MSX1* mutation was present in one out of the analyzed 34 Dutch patients with non-syndromic tooth agenesis. [8]

However, as previously mentioned, most mutations appeared to be present in *WNT10A*. Over 50 % of the cases carried a *WNT10A* mutation.

Hence, in **chapter 6**, we identified *MSX1* as a minor cause of hypodontia and *WNT10A* as a major gene in the etiology of hypodontia.

In **chapter 7**, we investigated the differences in the tooth crown morphology between patients with a *MSX1* nonsense mutation and non-affected age and gender matched controls, measured with a new developed 3D technique. Quantification of tooth crown shapes might contribute to early identification of *MSX1* related hypodontia.

In this study, significant differences in morphology were observed for the maxillary central incisor of the *MSX1* mutation carriers compared with the controls, including a larger volume. Strikingly, the *MSX1* cases presented with a more 'square' appearance.

The results, presented in **chapter 7** are in contrast with the finding that in hypodontia patients all developed teeth are in general smaller than in control groups. [63, 64]

We postulated that this tooth differences could be explained by the role of MSX1 in tooth morphogenesis. These identified differences in tooth morphology might differentiate *MSX1* hypodontia from other forms of hypodontia. This finding may contribute in clinical practice as well as in future studies.

MSX1 in relation to hydrocephaly

As previously mentioned, some *Msx1* mice mutants show, besides the craniofacial defect and tooth agenesis, a severe hydrocephalus with a collapse of the cerebral aqueduct (Chapter 1). [65, 66]

To the best of our knowledge, hydrocephaly has not been reported before in patients with a *MSX1* mutation. However, one can hypothesize this possible phenotype was not yet reported as result of a selection bias.

Therefore, the findings in mice motivated us to evaluate diagnostic results in patients with hydrocephaly due to an aqueduct stenosis examined for mutations in *MSX1*.

In **chapter 8**, we reported a *MSX*1 missense mutation found in a patient with hydrocephalus. The mutation is identified in a codon for a highly conserved amino acid located in the homeodomain of *MSX1*.

We realize that just a small serie of patients (n=9) was examined up until to date. Furthermore, the mutation identified is an unclassified variant and not all family members carrying the *MSX1* mutation showed signs associated with hydrocephaly. Hydrocephaly is a very heterogeneous complex and multifactorial disorder. [67] We therefore suggest that the role of *MSX1* in hydrocephaly in human deserves further study.

There is increasing evidence that genetic factors are involved in the etiology of hydrocephaly, the genetic causes of hydrocephaly in human patients remains to be elucidated. [68, 69] To date, only one gene causative for non-syndromic hydrocephaly in humans has been identified. [68]

A large number of genes are involved in numerous molecular pathways underlying congenital hydrocephalus, including *Msx1*. [68, 69] While *Msx1* is involved in the expression of diverse genes encoding proteins important during neural development [70], it is hypothesized that *Msx1* may also contributes to the expression of other genes involved in different molecular pathways (e.g. cell-cell adhesion, glycoprotein excretion) important in the etiology of hydrocephaly. [69]

As previously discussed, a combination of mutations in multiple genes might result in this specific phenotype. [71] *MSX1* might be one of the genes contributing to the risk of some forms of hydrocephaly.

Contribution to clinical practice

Genetic testing

In this thesis, we identified *MSX1* causative for autosomal dominant orofacial clefting in combination with hypodontia (**chapter 3**). In the family presented in the first lines of this thesis, we could identify a *MSX1* nonsense mutation, which was of major importance for accurate genetic counselling.

Based on this finding, *MSX1* analysis is to be considered in patients and families presenting with clefting and hypodontia.

In clinical practise, the presence of different cleft categories in a family might indicate a *MSX1* mutation is segregating in this family.

In addition, difference in tooth dimensions could contribute to an early identification of *MSX1* mutation and to assess the phenotypic diversity among carriers of a mutation. With a novel 3D technique significant shape differences in *MSX1* mutation carriers were observed (**chapter 7**). In particular the central incisor has a 'square' appearance, in contrast to most hypodontia patients in which all teeth are in general smaller.

Furthermore, we demonstrated that *MSX1* contributes in the etiology of non-syndromic clefting and isolated hypodontia in the Dutch population (**chapter 5 and chapter 6**). Although, we realize this contribution is small.

We demonstrated that *WNT10A* is a major gene in the etiology of hypodontia (**chapter 6**). By including *WNT10A* in the DNA diagnostics of isolated tooth agenesis, the cause of tooth agenesis can be identified in strikingly more patients. The yield of molecular testing in this condition was increased from 15% to 71%.

In the family introduced in the first lines of this chapter, a *WNT10A* mutation was identified in the patient with hypodontia. Interestingly, array CGH analysis in the brother of the patient, revealed a deletion of Xq21.1, including the genes *ITM2A*, *TBX22* and *BRWD3* (MRX93). The last two genes could explain respectively the cleft palate and the mental retardation in this brother.

To identify a genetic cause of tooth agenesis in patients with hypodontia, we recommend to test *WNT10A* in patients with an agenesis of six or more teeth, excluding the third molar, or in patients with a lower number of absent teeth and a positive family history for tooth agenesis, and/or ectodermal features.

In case *WNT10*A is negative for mutations, we recommend analysis of additional

hypodontia genes with a lower prevalence, i.c. *MSX1*, *PAX9* and *AXIN2*. In addition, analysis of *EDA*, *EDAR* and *EDARADD* should be considered in all cases with non-syndromic tooth agenesis of more than six teeth. With this approach, in more than 70% of the probands the underlying genetic factor can be identified. This will improve genetic counselling of patients with hypodontia and their families.

Counselling

Genetic counselling of patients with orofacial clefting and/or hypodontia starts with thorough clinical diagnostics, including accurate dysmorphologic investigation.
By identifying a genetic cause in patients with orofacial clefting and/or hypodontia a more tailored counselling can be provided. Identification of a syndrome can predict outcome, inheritance and recurrence risk.
When a *MSX1* mutation is identified in a family with orofacial clefting and hypodontia, *MSX1* mutation carriers should be aware of a possible increased orofacial cleft risk in their offspring. Furthermore, carrier testing is now available.

Until recently, *WNT10A* was associated with the diagnosis Odontoonychodermal dysplasia (OODD OMIM 257980). However, our study revealed that *WNT10A* mutations might be accompanied with isolated hypodontia (**chapter 6**).
From counselling perspective, it should be realized that the inheritance pattern of *WNT10A* mutations is less straightforward. Heterozygosity, compound heterozygosity and homozygosity were found to be associated with severe hypodontia, but also with milder features.

In addition, we found that in the Dutch population, periconceptional smoking by both parents may interact with a specific allelic variant of *MSX1* to significantly increase the orofacial cleft risk in their offspring. Our finding may encourage both prospecting mothers and fathers smoking to adjust their lifestyle and stop smoking during pregnancy.

In **chapter 2**, we found evidence that different cleft categories are variously associated with additional congenital anomalies and underlying chromosomal defects. Postnatal studies showed that cleft lip was less frequently associated with accompanying defects than cleft lip and palate and cleft palate. Based on this review, cases with cleft lip without associated anomalies have the most favourable prognosis concerning underlying chromosomal defects. Therefore, in these cases conventional karyotyping is not recommended. However, in all cleft categories, a deletion 22q11.2 and array CGH should be considered.

Future Prospects

New techniques

Although, we could increase the yield of genetic testing in hypodontia, the genetic cause could not be determined in all patients with hypodontia. Furthermore, we conclude in the minority of hypodontia and orofacial cleft cases *MSX1* is responsible for tooth agenesis and/or clefting.

Currently, advances in genetic technologies give new opportunities in identifying novel candidate genes for clefting and/or hypodontia. [72]

Array based methods enable to detect sub-microscopic chromosome imbalances. The results of array comparative genome hybridisation (Array CGH) in patients referred to our clinic and those of previous studies demonstrated that array CGH is a helpful diagnostic tool in some patients and/or families with orofacial celfting and/ or hypodontia. [73, 74] We could resolve the cause of orofacial cleft in combination with hypodontia in a few patients by identifying a deletion of respectively *TBX22*, *P63* and *IRF6*.

In addition, array based methods might reveal interesting novel candidate genes. Also, deletions and duplication in noncoding regions might be of importance. Variation in regulatory regions might influence the expression of a neighbouring gene and have phenotypic consequences. [74] Interestingly, three cases with Pierre Robin sequence and a deletion in the *SOX9* regulatory region have been reported. [74] Pierre Robin sequence is characterized by cleft palate, micrognathia cleft palate and glossoptosis. Often hypodontia is observed. In one patient with Pierre Robin sequence, a mutation in the enhancer of *SOX9* was identified, which in vivo altered the binding with MSX1. [75]

In conclusion, duplication and deletions and mutations in noncoding regions deserve further attention. Future studies might elucidate the role between distant regulators and their target genes [74] and reveal new pathogenetic mechanisms involved in congenital anomalies, including orofacial clefting and hypodontia. However, drawing causality of new findings will be a great challenge. The extensive functional information that was recently provided by the ENCODE project will be valuable in interpreting. [76]

Chromosome translocations have been of help in rare cases in the identification and/or confirmation of candidate genes for orofacial clefting. [77, 78] Nowadays, mate-pair sequencing might be an additional and useful approach. [79, 80] With mate-pair sequencing novel structural variations might be detected, which could be used to identify candidate genes for orofacial clefting and tooth agenesis. [80]

With next generation sequencing, the entire protein-coding sequence (the exome) or even the entire human genome can be sequenced. This rapidly developing technique has been very successful in identifying disease genes. [81] Also, in patients with orofacial clefting and/or hypodontia and in families presenting more affected family members next generation sequencing might identify causative variants.

However, it should be realized that accurate phenotyping and sub-phenotyping are important in the search for novel candidate genes. [82, 83]

The differences in prevalence of additional anomalies between the different cleft categories reported in **chapter 2**, which reflects the differences in etiology and development, also demonstrate the significance of defining different cleft categories.

We suggest that sub-classifications should be made by integrating embryology, developmental mechanisms, molecular pathways a well as clinical experience. In this perspective, subdividing of orofacial clefting in apoptosis and/or differentiation/ outgrowth defects could be considered. Based on more recent studies [83- 85] and because different cleft categories are variously associated with accompanying anomalies and chromosomal defects (**chapter 2**), orofacial clefts should preferably be sub-classified in cleft lip/ alveolus only, cleft lip/alveolus and palate, and cleft palate only. Moreover, sub-classifying cleft lip in incomplete and complete cleft lip might be considered. Furthermore, specific dysmorphic features (e.g. hypertelorism vs. hypotelorism) should be taken in to consideration in the sub-classification. [85]

In addition, accurate dental phenotyping, including evaluation of tooth dimensions in 3-dimensional manner as discussed in **chapter 7**, could contribute to sub-phenotyping of patients with hypodontia and clefting in further research. Although, we realize the technique is time consuming at the moment. Further software development should make the method more user-friendly and applicable for future studies.

Gene regulation: Sense- Antisense RNA

As discussed above, variants in non-coding regions might alter gene- expression. In **chapter 4** we discussed that the formation of both a sense (S) and an antisense (AS) RNA play a role in the expression of the MSX1 protein. The AS RNA was shown to inhibit *Msx1* expression by decreasing the half-life of the corresponding S RNA. [86] Therefore, in mouse, the ratio of both *Msx1* RNAs is supposed to be a key factor for cell differentiation and plays a role in phenotypic expression in mineralized tissues. [87]

We postulated that deleterious mutations in the *MSX1* gene might have an effect on the phenotype by influencing the level of S RNA and thus disrupting the balance between S

and AS RNA. This altered balance might explain the orofacial clefting in the p. Ser111**MSX1* family we identified.

We studied the *MSX1* S and AS RNA transcripts in our family. This study showed that in skin *MSX1* S and AS RNAs were both present, and the nonsense mutation results in nonsense mediated mRNA decay. As NMD due to p.Ser111* lowers the ratio of the *MSX1* S and AS RNA, it is tentative to suggest that the relatively increased level of AS RNA as compared to the S RNA of the normal allele has an additive dominant negative effect on the S RNA of the normal allele. This might result in a more severe phenotype and the presence of clefting in this particular family. However, it is necessary to identify and study more families with *MSX1* mutations to further elucidate the genotype – phenotype relationship. Furthermore, one should realize that the ratio of both RNA's might differ between tissues and in different stages of pre- and postnatal development.

Animal models: Zebrafish

Next generation sequencing will enable to detect a large number of novel variants in novel genes. However, to interpret the relevance of these variants in the etiology of developmental disorder will be a major challenge. An increasing number of studies demonstrated that zebra fish, beside mouse models, are quite resourceful in identifying and functional validating candidate genes involved in human disorders. [78, 88 – 91]

Interestingly, a zebrafish knockdown study supported the role of *FAF1* in the etiology of cleft palate in a boy with cleft of the secondary palate and associated micro/retrognathia, microstomia, malar hypoplasia, and hypodontia. [78] Knockdown of zebrafish *faf1* leads to pharyngeal cartilage defects and jaw abnormality, with deficient expression of *sox9a* and *col2a1*. [78] As mentioned above, a previous study reported a patient with Pierre Robin sequence with a mutation in the enhancer of *SOX9*, which in vivo altered the binding with MSX1. [75] To the best of our knowledge, a possible relation of *FAF1* and *MSX1* in common molecular pathways remains to be elucidated.

A more recent zebrafish study demonstrated that knockdown of *Crispld2* result in significant jaw and palatal abnormalities, supporting the involvement of *Crispld2* in the etiology of non-syndromic orofacial clefting. [92]

Both studies illustrate that zebrafish might be valuable in elucidating genetic factors involved in orofacial clefting.

From a different perspective: Cilia

The last years, increasing evidence becomes available for the significant role of cilia in craniofacial development. [93, 94]

Cilia are antenna-like structures that extend from the apical surface of many growth-arrested or differentiated cells. [95-97] The primary non-motile cilium participates in several signalling pathways including the SHH, WNT, PDGF and FGF signalling pathways, [96, 97] which are also important in craniofacial and tooth development (see also Chapter 1). [93, 94, 98] Cilia are important for cell growth and cell adhesion. Furthermore, interaction with the extra cellular matrix (e.g. collagen) is suggested. [99]

Interestingly, cilia are also identified on odontoblasts, suggesting a role of cilia in tooth development as well. [100, 101] It has been demonstrated that primary cilia regulate *Shh* activity and regulates tooth number and morpogenesis [102] Interestingly, as previously discussed in **chapter 4** also MSX1 is involved in *Shh* signalling and *wnt*-signalling.

To date, a number of ciliopathies associated with orofacial clefting and/or hypodontia have been identified, including Oro-facial-Digital syndrome 1 and 4 (resp. OMIM 311200/ 258860), Bardet-Biedl syndrome (OMIM 209900) and Cranioectodermal Dysplasia (CED) (OMIM 218330). [94, 103, 104]

Based on the above, we suggest that ciliopathy is an interesting field of future research in (non-syndromic) clefting and dental anomalies. It will be of help that the zebrafish appears to be a useful organism to investigate the consequences of ciliary dysfunction. [105]

Hypodontia and cleft: relation to cancer?

In **chapter 5** we identified a mutation in *AXIN2* associated with hypodontia and colon cancer, as previously reported by Lammi et al. [106] supporting a relation between hypodontia and cancer. Several studies have been suggestive for a relation between hypodontia and cancer.

Also, Basal Cell Nevus (Gorlin) syndrome (BCNS, OMIM 109400) caused by mutations in *PTCH1* (discussed in **chapter 1**) is associated with cleft, hypodontia and cancer.

Furthermore, a study in patients with epithelial ovarian cancer (EOC) and control subjects revealed that women with EOC are 8.1 times more likely to have hypodontia than are women without EOC. [107]

Recently, a Dutch study supported the association of clefts and developing gastric cancer due to mutations in *CDH1* as previously reported. [108, 109]

A recent epidemiological study revealed that individuals born with CL/P and their family

members have a higher prevalence of cancer than the general population. [110]
These studies support the hypothesis that genes involved in the etiology of clefting and hypodontia may be involved in cancer development. [111] More studies are needed to be adequate about the suggested relation between hypodontia, cleft and cancer.

Multidisciplinary approach and collaboration

Orofacial clefting and hypodontia are both complex, heterogeneous congenital anomalies. To reveal new insights in the etiology in clefting and hypodontia an integrated approach is of great importance. Therefore, we encourage developmental biologists, medical geneticists, bio-informaticians and clinicians to join forces in future research, which might give new opportunities. Prioritizing candidate genes and as already mentioned, interpretation of mutations identified with the above-discussed advanced molecular techniques is a major challenge.

As proposed, subphenotyping might contribute in identifying new cleft and hypodontia genes or modifiers. Therefore, a large number of patients are required, and a careful clinical assessment has to be made. While some symptoms and syndromes might be diagnosed later in infancy, a prospective study design is preferred.
In addition, rare syndromic cases might reveal new genetic factors contributing in the etiology of cleft and hypodontia.
To recruit these specific patient-cohorts multidisciplinary teams, and national and international collaboration are essential.

Our collaboration with the Department of Prosthodontics and Special Dental Care has enabled us to reveal *WNT10A* as a major gene in the etiology of hypodontia. The cause of the hypodontia could not be identified in all hypodontia cases that were included in this study. This thoroughly phenotyped cohort is unique and will be valuable for additional studies.
In collaboration with the Department of Plastic and Reconstructive Surgery, Department of Oral and Maxillofacial Surgery and Prosthodontics and Special Dental Care, research on the specific cleft categories (e.g. Pierre Robin Sequence) may reveal new insights in the etiology of hypodontia, orofacial clefting and craniofacial disorders. Understanding of the etiology is significant for a more tailored genetic counseling and might be a first step in contributing to personalized treatment.

References

1. Vieira AR. Genetic and environmental factors in human cleft lip and palate. Front Oral Biol. 2012;16:19-31.
2. Dixon MJ, Marazita ML, Beaty TH, Murray JC. Cleft lip and palate: understanding genetic and environmental influences. Nat Rev Genet. 2011;12:167-78. Review.
3. Polder BJ, Van't Hof MA, Van der Linden FP, Kuijpers-Jagtman AM. A meta-analysis of the prevalence of dental agenesis of permanent teeth. Community Dent Oral Epidemiol 2004; 32: 217-226.
4. Grosen D, Chevrier C, Skytthe A, Bille C, Mølsted K, Sivertsen A, Murray JC, Christensen K. A cohort study of recurrence patterns among more than 54,000 relatives of oral cleft cases in Denmark: support for the multifactorial threshold model of inheritance. J Med Genet. 2010;47:162-8.
5. Bailleul-Forestier I, Molla M, Verloes A, Berdal A. The genetic basis of inherited anomalies of the teeth. Part 1: clinical and molecular aspects of non-syndromic dental disorders. Eur J Med Genet. 2008;51:273-91
6. Shafi I, Phillips JM, Dawson MP, Broad RD, Hosey MT. A study of patients attending a multidisciplinary hypodontia clinic over a five year period. Br Dent J. 2008;205:649-52; discussion 646.
7. Nieminen P. Genetic basis of tooth agenesis. J Exp Zool B Mol Dev Evol. 2009;312B:320-42.
8. van den Boogaard MJ, Créton M, Bronkhorst Y, van der Hout A, Hennekam E, Lindhout D, Cune M, Ploos van Amstel HK. Mutations in WNT10A are present in more than half of isolated hypodontia cases. J Med Genet. 2012;49:327-31.
9. Bergendal B, Klar J, Stecksén-Blicks C, Norderyd J, Dahl N. Isolated oligodontia associated with mutations in EDARADD, AXIN2, MSX1, and PAX9 genes. Am J Med Genet A. 2011;155A:1616-22.
10. Maarse W, Rozendaal AM, Pajkrt E, Vermeij-Keers C, Mink van der Molen AB, van den Boogaard MJ. A systematic review of associated structural and chromosomal defects in oral clefts: when is prenatal genetic analysis indicated? J Med Genet. 2012;49:490-8.
11. Pedro RL, Faria MD, Costa MC, Vieira AR. Dental Anomalies in Children Born with Clefts: A Case-Control Study. Cleft Palate Craniofac J. 2011 Jul 8. [Epub ahead of print]
12. Tannure PN, Oliveira CA, Maia LC, Vieira AR, Granjeiro JM, de Castro Costa M. Prevalence of Dental Anomalies in Nonsyndromic Individuals With Cleft Lip and Palate: A Systematic Review and Meta-analysis. Cleft Palate Craniofac J. 2012;49:194-200.
13. Yildirim M, Seymen F, Deeley K, Cooper ME, Vieira AR. Defining predictors of cleft lip and palate risk. J Dent Res. 2012;91:556-61.
14. Küchler EC, da Motta LG, Vieira AR, Granjeiro JM. Side of dental anomalies and taurodontism as potential clinical markers for cleft subphenotypes. Cleft Palate Craniofac J. 2011;48:103-8.
15. http://OMIM.org
16. London Medical Databases ; http://www.lmdatabases.com/
17. Satokata I, Maas R. Msx1 deficient mice exhibit cleft palate and abnormalities of craniofacial and tooth development. Nat Genet. 1994;6:348-56.

18. Mackenzie A, Leeming GL, Jowett AK, Ferguson MW, Sharpe PT. The homeobox gene Hox 7.1 has specific regional and temporal expression patterns during early murine craniofacial embryogenesis, especially tooth development in vivo and in vitro. Development. 1991;111:269-85.
19. MacKenzie A, Ferguson MW, Sharpe PT. Hox-7 expression during murine craniofacial development. Development. 1991;113:601-11.
20. Lidral AC, Romitti PA, Basart AM, Doetschman T, Leysens NJ, Daack-Hirsch S, Semina EV, Johnson LR, Machida J, Burds A, Parnell TJ, Rubenstein JL, Murray JC. Association of MSX1 and TGFB3 with nonsyndromic clefting in humans. Am J Hum Genet. 1998;63:557-68.
21. van den Boogaard MJ, Dorland M, Beemer FA, van Amstel HK. MSX1 mutation is associated with orofacial clefting and tooth agenesis in humans. Nat Genet. 2000;24:342-3.
22. Jezewski PA, Vieira AR, Nishimura C, Ludwig B, Johnson M, O'Brien SE, Daack-Hirsch S, Schultz RE, Weber A, Nepomucena B, Romitti PA, Christensen K, Orioli IM, Castilla EE, Machida J, Natsume N, Murray JC. Complete sequencing shows a role for MSX1 in non-syndromic cleft lip and palate. J Med Genet. 2003;40:399-407.
23. Romitti PA, Lidral AC, Munger RG, Daack-Hirsch S, Burns TL, Murray JC. Candidate genes for nonsyndromic cleft lip and palate and maternal cigarette smoking and alcohol consumption: evaluation of genotype-environment interactions from a population-based case-control study of orofacial clefts. Teratology. 1999;59:39-50.
24. Jugessur A, Lie RT, Wilcox AJ, Murray JC, Taylor JA, Saugstad OD, Vindenes HA, Abyholm F. Variants of developmental genes (TGFA, TGFB3, and MSX1) and their associations with orofacial clefts: a case-parent triad analysis. Genet Epidemiol. 2003;24:230-9.
25. Vieira AR, Orioli IM, Castilla EE, Cooper ME, Marazita ML, Murray JC. MSX1 and TGFB3 contribute to clefting in South America. J Dent Res. 2003;82:289-92.
26. Moreno LM, Arcos-Burgos M, Marazita ML, Krahn K, Maher BS, Cooper ME, Valencia-Ramirez CR, Lidral AC. Genetic analysis of candidate loci in non-syndromic cleft lip families from Antioquia-Colombia and Ohio. Am J Med Genet A. 2004;125A:135-44.
27. Schultz RE, Cooper ME, Daack-Hirsch S, Shi M, Nepomucena B, Graf KA, O'Brien EK, O'Brien SE, Marazita ML, Murray JC. Targeted scan of fifteen regions for nonsyndromic cleft lip and palate in Filipino families. Am J Med Genet A. 2004;125A:17-22.
28. Suazo J, Santos JL, Carreño H, Jara L, Blanco R. Linkage disequilibrium between MSX1 and non-syndromic cleft lip/palate in the Chilean population. J Dent Res. 2004;83:782-5.
29. Suzuki Y, Jezewski PA, Machida J, Watanabe Y, Shi M, Cooper ME, Viet le T, Nguyen TD, Hai H, Natsume N, Shimozato K, Marazita ML, Murray JC. In a Vietnamese population, MSX1 variants contribute to cleft lip and palate. Genet Med. 2004;6:117-25.
30. Tongkobpetch S, Siriwan P, Shotelersuk V. MSX1 mutations contribute to nonsyndromic cleft lip in a Thai population. J Hum Genet. 2006;51:671-6.
31. Otero L, Gutiérrez S, Cháves M, Vargas C, Bérmudez L. Association of MSX1 with nonsyndromic cleft lip and palate in a Colombian population. Cleft Palate Craniofac J. 2007;44:653-6.
32. Park J, Park BY, Kim HS, Lee JE, Suh I, Nam CM, Kang DR, Kim S, Yun JE, Go EN, Jee SH, Beaty TH. MSX1 polymorphism associated with risk of oral cleft in Korea: evidence from case-parent trio and case-control studies.Yonsei Med J. 2007;48:101-8.
33. Ingersoll RG, Hetmanski J, Park JW, Fallin MD, McIntosh I, Wu-Chou YH, Chen PK, Yeow V, Chong SS, Cheah F, Sull JW, Jee SH, Wang H, Wu T, Murray T, Huang S, Ye X, Jabs EW, Redett R, Raymond G, Scott AF, Beaty TH. Association between genes on chromosome 4p16 and non-syndromic oral clefts in four populations. Eur J Hum Genet. 2010;18:726-32.

34. Jagomägi T, Nikopensius T, Krjutskov K, Tammekivi V, Viltrop T, Saag M, Metspalu A. MTHFR and MSX1 contribute to the risk of nonsyndromic cleft lip/palate. Eur J Oral Sci. 2010;118:213-20.

35. Nikopensius T, Jagomägi T, Krjutskov K, Tammekivi V, Saag M, Prane I, Piekuse L, Akota I, Barkane B, Krumina A, Ambrozaityte L, Matuleviciene A, Kucinskiene ZA, Lace B, Kucinskas V, Metspalu A. Genetic variants in COL2A1, COL11A2, and IRF6 contribute risk to nonsyndromic cleft palate. Birth Defects Res A Clin Mol Teratol. 2010;88:748-56.

36. Liang J, Zhu L, Meng L, Chen D, Bian Z. Novel nonsense mutation in MSX1 causes tooth agenesis with cleft lip in a Chinese family. Eur J Oral Sci. 2012;120:278-82

37. Vieira AR, Modesto A, Meira R, Barbosa AR, Lidral AC, Murray JC. Interferon regulatory factor 6 (IRF6) and fibroblast growth factor receptor 1 (FGFR1) contribute to human tooth agenesis. Am J Med Genet A. 2007;143:538-45.

38. Murray JC, Schutte BC. Cleft palate: players, pathways, and pursuits. J Clin Invest. 2004;113:1676-8.

39. Alappat S, Zhang ZY, Chen YP. Msx homeobox gene family and craniofacial development. Cell Res. 2003;13:429-42. Review.

40. Zhang Z, Song Y, Zhao X, Zhang X, Fermin C, Chen Y. Rescue of cleft palate in Msx1-deficient mice by transgenic Bmp4 reveals a network of BMP and Shh signaling in the regulation of mammalian palatogenesis. Development. 2002;129:4135-46.

41. Han J, Ito Y, Yeo JY, Sucov HM, Maas R, Chai Y. Cranial neural crest-derived mesenchymal proliferation is regulated by Msx1-mediated p19(INK4d) expression during odontogenesis. Dev Biol. 2003;261:183-96.

42. De Coster PJ, Marks LA, Martens LC, Huysseune A. Dental agenesis: genetic and clinical perspectives. J Oral Pathol Med. 2009;38:1-17.

43. Chen Y, Bei M, Woo I, Satokata I, Maas R. Msx1 controls inductive signaling in mammalian tooth morphogenesis. Development. 1996;122:3035-44.

44. Orestes-Cardoso S, Nefussi JR, Lezot F, Oboeuf M, Pereira M, Mesbah M, Robert B, Berdal A. Msx1 is a regulator of bone formation during development and postnatal growth: in vivo investigations in a transgenic mouse model. Connect Tissue Res. 2002;43:153-60.

45. Zhang Z, Song Y, Zhang X, Tang J, Chen J, Chen Y. Msx1/Bmp4 genetic pathway regulates mammalian alveolar bone formation via induction of Dlx5 and Cbfa1. Mech Dev. 2003;120:1469-79.

46. Han J, Mayo J, Xu X, Li J, Bringas P Jr, Maas RL, Rubenstein JL, Chai Y. Indirect modulation of Shh signaling by Dlx5 affects the oral-nasal patterning of palate and rescues cleft palate in Msx1-null mice. Development. 2009;136:4225-33.

47. Suzuki S, Marazita ML, Cooper ME, Miwa N, Hing A, Jugessur A, Natsume N, Shimozato K, Ohbayashi N, Suzuki Y, Niimi T, Minami K, Yamamoto M, Altannamar TJ, Erkhembaatar T, Furukawa H, Daack-Hirsch S, L'heureux J, Brandon CA, Weinberg SM, Neiswanger K, Deleyiannis FW, de Salamanca JE, Vieira AR, Lidral AC, Martin JF, Murray JC. Mutations in BMP4 are associated with subepithelial, microform, and overt cleft lip. Am J Hum Genet. 2009;84:406-11

48. Cobourne MT, Sharpe PT. Making up the numbers: The molecular control of mammalian dental formula. Semin Cell Dev Biol. 2010;21:314-24

49. Yamashiro T, Zheng L, Shitaku Y, Saito M, Tsubakimoto T, Takada K, Takano-Yamamoto T, Thesleff I. Wnt10a regulates dentin sialophosphoprotein mRNA expression and possibly links odontoblast differentiation and tooth morphogenesis. Differentiation. 2007;75:452-62.

50. Adaimy L, Chouery E, Megarbane H, Mroueh S, Delague V, Nicolas E, Belguith H, de Mazancourt P, Megarbane A. Mutation in WNT10A is associated with an autosomal recessive ectodermal dysplasia: the odonto-onycho-dermal dysplasia. Am J Hum Genet. 2007;81:821-8.
51. Bohring A, Stamm T, Spaich C, Haase C, Spree K, Hehr U, Hoffmann M, Ledig S, Sel S, Wieacker P, Röpke A. WNT10A mutations are a frequent cause of a broad spectrum of ectodermal dysplasias with sex-biased manifestation pattern in heterozygotes. Am J Hum Genet. 2009;85:97-105.
52. Kantaputra P, Sripathomsawat W. WNT10A and isolated hypodontia. Am J Med Genet A. 2011;155A:1119-22.
53. Vieira AR. Unraveling human cleft lip and palate research. J Dent Res. 2008;87:119-25. Review.
54. Shi M, Wehby GL, Murray JC. Review on genetic variants and maternal smoking in the etiology of oral clefts and other birth defects. Birth Defects Res C Embryo Today. 2008;84:16-29. Review.
55. Beaty TH, Hetmanski JB, Zeiger JS, Fan YT, Liang KY, VanderKolk CA, McIntosh I. Testing candidate genes for non-syndromic oral clefts using a case-parent trio design. Genet Epidemiol. 2002;22:1-11.
56. Chevrier C, Bahuau M, Perret C, Iovannisci DM, Nelva A, Herman C, Vazquez MP, Francannet C, Robert-Gnansia E, Lammer EJ, Cordier S. Genetic susceptibilities in the association between maternal exposure to tobacco smoke and the risk of nonsyndromic oral cleft. Am J Med Genet A. 2008;146A:2396-406.
57. van den Boogaard MJ, de Costa D, Krapels IP, Liu F, van Duijn C, Sinke RJ, Lindhout D, Steegers-Theunissen RP. The MSX1 allele 4 homozygous child exposed to smoking at periconception is most sensitive in developing nonsyndromic orofacial clefts. Hum Genet. 2008;124:525-34.
58. Infante-Rivard C, Jacques L. Empirical study of parental recall bias. Am J Epidemiol. 2000 Sep 1;152:480-6.
59. Little J, Cardy A, Munger RG. Tobacco smoking and oral clefts: a meta-analysis. Bull World Health Organ. 2004;82:213-8.
60. Little J, Cardy A, Arslan MT, Gilmour M, Mossey PA; United Kingdom-based case-control study. Smoking and orofacial clefts: a United Kingdom-based case-control study. Cleft Palate Craniofac J. 2004;41:381-6.
61. Li Z, Liu J, Ye R, Zhang L, Zheng X, Ren A. Maternal passive smoking and risk of cleft lip with or without cleft palate. Epidemiology. 2010;21:240-2.
62. Zhang B, Jiao X, Mao L, Xue J. Maternal cigarette smoking and the associated risk of having a child with orofacial clefts in China: a case-control study. J Craniomaxillofac Surg. 2011;39:313-8.
63. Brook AH, Elcock C, al-Sharood MH, McKeown HF, Khalaf K, Smith RN. Further studies of a model for the etiology of anomalies of tooth number and size in humans. Connect Tissue Res. 2002;43:289-95.
64. McKeown HF, Robinson DL, Elcock C, al-Sharood M, Brook AH. Tooth dimensions in hypodontia patients, their unaffected relatives and a control group measured by a new image analysis system. Eur J Orthod. 2002;24:131-41.

65. Fernández-Llebrez P, Grondona JM, Pérez J, López-Aranda MF, Estivill-Torrús G, Llebrez-Zayas PF, Soriano E, Ramos C, Lallemand Y, Bach A, Robert B. Msx1-deficient mice fail to form prosomere 1 derivatives, subcommissural organ, and posterior commissure and develop hydrocephalus. J Neuropathol Exp Neurol. 2004;63:574-86.

66. Ramos C, Fernández-Llebrez P, Bach A, Robert B, Soriano E. Msx1 disruption leads to diencephalon defects and hydrocephalus. Dev Dyn. 2004;230:446-60.

67. Verhagen JM, Schrander-Stumpel CT, Krapels IP, de Die-Smulders CE, van Lint FH, Willekes C, Weber JW, Gavilanes AW, Macville MV, Stegmann AP, Engelen JJ, Bakker J, Vos YJ, Frints SG. Congenital hydrocephalus in clinical practice: a genetic diagnostic approach. Eur J Med Genet. 2011;54:e542-7.

68. Zhang J, Williams MA, Rigamonti D. Genetics of human hydrocephalus. J Neurol. 2006;253:1255-66.

69. Huh MS, Todd MA, Picketts DJ. SCO-ping out the mechanisms underlying the etiology of hydrocephalus. Physiology (Bethesda). 2009;24:117-26. Review.

70. Ramos C, Robert B. msh/Msx gene family in neural development. Trends Genet. 2005;21:624-32.

71. Picketts D.J. Neuropeptide signaling and hydrocephalus: SCO with the flow The J. of Clin Invest. 2006;116:1828-1832

72. Rahimov F, Jugessur A, Murray JC. Genetics of nonsyndromic orofacial clefts. Cleft Palate Craniofac J. 2012;49:73-91.

73. Shi M, Mostowska A, Jugessur A, Johnson MK, Mansilla MA, Christensen K, Lie RT, Wilcox AJ, Murray JC. Identification of microdeletions in candidate genes for cleft lip and/or palate. Birth Defects Res A Clin Mol Teratol. 2009;85:42-51.

74. Klopocki E, Mundlos S. Copy-number variations, noncoding sequences, and human phenotypes. Annu Rev Genomics Hum Genet. 2011;12:53-72.

75. Benko S, Fantes JA, Amiel J, Kleinjan DJ, Thomas S, Ramsay J, Jamshidi N, Essafi A, Heaney S, Gordon CT, McBride D, Golzio C, Fisher M, Perry P, Abadie V, Ayuso C, Holder-Espinasse M, Kilpatrick N, Lees MM, Picard A, Temple IK, Thomas P, Vazquez MP, Vekemans M, Roest Crollius H, Hastie ND, Munnich A, Etchevers HC, Pelet A, Farlie PG, Fitzpatrick DR, Lyonnet S. Highly conserved non-coding elements on either side of SOX9 associated with Pierre Robin sequence. Nat Genet. 2009 Mar;41(3):359-64

76. Ecker JR, Bickmore WA, Barroso I, Pritchard JK, Gilad Y, Segal E. Genomics: ENCODE explained. Nature. 2012;489:52-5.

77. Machida J, Félix TM, Murray JC, Yoshiura K, Tanemura M, Kamamoto M, Shimozato K, Sonta S, Ono T. Searching for genes for cleft lip and/or palate based on breakpoint analysis of a balanced translocation t(9;17)(q32;q12). Cleft Palate Craniofac J. 2009;46:532-40.

78. Ghassibe-Sabbagh M, Desmyter L, Langenberg T, Claes F, Boute O, Bayet B, Pellerin P, Hermans K, Backx L, Mansilla MA, Imoehl S, Nowak S, Ludwig KU, Baluardo C, Ferrian M, Mossey PA, Noethen M, Dewerchin M, François G, Revencu N, Vanwijck R, Hecht J, Mangold E, Murray J, Rubini M, Vermeesch JR, Poirel HA, Carmeliet P, Vikkula M. FAF1, a gene that is disrupted in cleft palate and has conserved function in zebrafish. Am J Hum Genet. 2011;88:150-61.

79. Korbel JO, Urban AE, Affourtit JP, Godwin B, Grubert F, Simons JF, Kim PM, Palejev D, Carriero NJ, Du L, Taillon BE, Chen Z, Tanzer A, Saunders AC, Chi J, Yang F, Carter NP, Hurles ME, Weissman SM, Harkins TT, Gerstein MB, Egholm M, Snyder M. Paired-end mapping reveals extensive structural variation in the human genome. Science. 2007;318:420-6.

80. Vergult S, Van Binsbergen E, Sante T, Nowak S, Vanakker O, Janssens S, Claes K, Poppe B,Van der Aa N, Markus J. van Roosmalen M, Duran K,Tavakoli-Yaraki M, Swinkels, van den Boogaard, van Haelst M, Roelens F,4 De Paepe A, Speleman F, Cuppen E, Mortier G, Kloosterman G, Menten B Mate pair sequencing for the detection of balanced and unbalanced chromosomal aberrations in patients with intellectual disability and congenital malformations- manuscript in preparation
81. Gilissen C, Hoischen A, Brunner HG, Veltman JA. Disease gene identification strategies for exome sequencing. Eur J Hum Genet. 2012;20:490-7.
82. Hennekam RC, Biesecker LG. Next-generation sequencing demands next-generation phenotyping. Hum Mutat. 2012;33:884-6.
83. Rahimov F, Jugessur A, Murray JC. Genetics of nonsyndromic orofacial clefts. Cleft Palate Craniofac J. 2012;49:73-91.
84. Luijsterburg AJ, Vermeij-Keers C. Ten years recording common oral clefts with a new descriptive system. Cleft Palate Craniofac J. 2011;48:173-82.
85. Vermeij-keers C, Rozendaal AM, van den Boogaard M-JH , Mink van der molen AB, Pajkrt E New ultrasound classification of oral and craniofacial clefts based on embryology. Manuscript in preparation.
86. Petit S, Meary F, Pibouin L, Jeanny JC, Fernandes I, Poliard A, Hotton D, Berdal A, Babajko S. Autoregulatory loop of Msx1 expression involving its antisense transcripts. J Cell Physiol. 2009;220:303-10.
87. Babajko S, Petit S, Fernandes I, Méary F, LeBihan J, Pibouin L, Berdal A. Msx1 expression regulation by its own antisense RNA: consequence on tooth development and bone regeneration. Cells Tissues Organs. 2009;189:115-21
88. Manzini MC, Tambunan DE, Hill RS, Yu TW, Maynard TM, Heinzen EL, Shianna KV, Stevens CR, Partlow JN, Barry BJ, Rodriguez J, Gupta VA, Al-Qudah AK, Eyaid WM, Friedman JM, Salih MA, Clark R, Moroni I, Mora M, Beggs AH, Gabriel SB, Walsh CA. Exome Sequencing and Functional Validation in Zebrafish Identify GTDC2 Mutations as a Cause of Walker-Warburg Syndrome. Am J Hum Genet. 2012;91:541-7.
89. Karpanen T, Schulte-Merker S. Zebrafish provides a novel model for lymphatic vascular research. Methods Cell Biol. 2011;105:223-38.
90. Alders M, Hogan BM, Gjini E, Salehi F, Al-Gazali L, Hennekam EA, Holmberg EE, Mannens MM, Mulder MF, Offerhaus GJ, Prescott TE, Schroor EJ, Verheij JB, Witte M, Zwijnenburg PJ, Vikkula M, Schulte-Merker S, Hennekam RC. Mutations in CCBE1 cause generalized lymph vessel dysplasia in humans. Nat Genet. 2009;41:1272-4.
91. Amsterdam A, Hopkins N. Mutagenesis strategies in zebrafish for identifying genes involved in development and disease. Trends Genet. 2006;22:473-8.
92. Yuan Q, Chiquet BT, Devault L, Warman ML, Nakamura Y, Swindell EC, Hecht JT. Craniofacial abnormalities result from knock down of nonsyndromic clefting gene, crispld2, in zebrafish. Genesis. 2012 Aug 6. doi: 10.1002/dvg.22051.
93. Brugmann SA, Cordero DR, Helms JA. Craniofacial ciliopathies: A new classification for craniofacial disorders. Am J Med Genet A. 2010;152A:2995-3006. Review.
94. Zaghloul NA, Brugmann SA. The emerging face of primary cilia. Genesis. 2011;49:231-46. Review.
95. Pedersen LB, Rosenbaum JL. Intraflagellar transport (IFT) role in ciliary assembly, resorption and signalling. Curr Top Dev Biol. 2008;85:23-61. Review.

96. Oh EC, Katsanis N. Cilia in vertebrate development and disease. Development. 2012;139:443-8. Review.

97. Veland IR, Awan A, Pedersen LB, Yoder BK, Christensen ST. Primary cilia and signaling pathways in mammalian development, health and disease. Nephron Physiol. 2009;111:p39-53. Review.

98. Xu X, Bringas P Jr, Soriano P, Chai Y. PDGFR-alpha signaling is critical for tooth cusp and palate morphogenesis. Dev Dyn. 2005;232:75-84.

99. Seeger-Nukpezah T, Golemis EA. The extracellular matrix and ciliary signaling. Curr Opin Cell Biol. 2012 Jul 19. [Epub ahead of print]

100. Magloire H, Couble ML, Romeas A, Bleicher F. Odontoblast primary cilia: facts and hypotheses. Cell Biol Int. 2004;28:93-9. Review.

101. Ohazama A, Haycraft CJ, Seppala M, Blackburn J, Ghafoor S, Cobourne M, Martinelli DC, Fan CM, Peterkova R, Lesot H, Yoder BK, Sharpe PT. Primary cilia regulate Shh activity in the control of molar tooth number. Development. 2009;136:897-903.

102. Thivichon-Prince B, Couble ML, Giamarchi A, Delmas P, Franco B, Romio L, Struys T, Lambrichts I, Ressnikoff D, Magloire H, Bleicher F. Primary cilia of odontoblasts: possible role in molar morphogenesis. J Dent Res. 2009;88:910-5.

103. Thomas S, Legendre M, Saunier S, Bessières B, Alby C, Bonnière M, Toutain A, Loeuillet L, Szymanska K, Jossic F, Gaillard D, Yacoubi MT, Mougou-Zerelli S, David A, Barthez MA, Ville Y, Bole-Feysot C, Nitschke P, Lyonnet S, Munnich A, Johnson CA, Encha-Razavi F, Cormier-Daire V, Thauvin-Robinet C, Vekemans M, Attié-Bitach T. TCTN3 Mutations Cause Mohr-Majewski Syndrome. Am J Hum Genet. 2012;91:372-8.

104. Bacino CA, Dhar SU, Brunetti-Pierri N, Lee B, Bonnen PE. WDR35 mutation in siblings with Sensenbrenner syndrome: A ciliopathy with variable phenotype. Am J Med Genet A. 2012 Sep 17

105. Zaghloul NA, Katsanis N. Zebrafish assays of ciliopathies. Methods Cell Biol. 2011;105:257-72.

106. Lammi L, Arte S, Somer M, Jarvinen H, Lahermo P, Thesleff I, Pirinen S, Nieminen P. Mutations in AXIN2 cause familial tooth agenesis and predispose to colorectal cancer. Am J Hum Genet. 2004;74:1043-50.

107. Chalothorn LA, Beeman CS, Ebersole JL, Kluemper GT, Hicks EP, Kryscio RJ, DeSimone CP, Modesitt SC. Hypodontia as a risk marker for epithelial ovarian cancer: a case-controlled study. J Am Dent Assoc. 2008;139:163-9.

108. Kluijt I, Siemerink EJ, Ausems MG, van Os TA, de Jong D, Simões-Correia J, van Krieken JH, Ligtenberg MJ, Figueiredo J, van Riel E, Sijmons RH, Plukker JT, van Hillegersberg R, Dekker E, Oliveira C, Cats A, Hoogerbrugge N; Dutch Working Group on Hereditary Gastric Cancer. CDH1-related hereditary diffuse gastric cancer syndrome: clinical variations and implications for counseling. Int J Cancer. 2012;131:367-76.

109. Frebourg T, Oliveira C, Hochain P, Karam R, Manouvrier S, Graziadio C, Vekemans M, Hartmann A, Baert-Desurmont S, Alexandre C, Lejeune Dumoulin S, Marroni C, Martin C, Castedo S, Lovett M, Winston J, Machado JC, Attié T, Jabs EW, Cai J, Pellerin P, Triboulet JP, Scotte M, Le Pessot F, Hedouin A, Carneiro F, Blayau M, Seruca R. Cleft lip/palate and CDH1/E-cadherin mutations in families with hereditary diffuse gastric cancer. J Med Genet. 2006;43:138-42.

110. Vieira AR, Khaliq S, Lace B. Risk of cancer in relatives of children born with isolated cleft lip and palate. Am J Med Genet A. 2012;158A:1503-4.

111. Andrade Filho PA, Letra A, Cramer A, Prasad JL, Garlet GP, Vieira AR, Ferris RL, Menezes R. Insights from studies with oral cleft genes suggest associations between WNT-pathway genes and risk of oral cancer. J Dent Res. 2011;90:740-6.

Nederlandse samenvatting (Dutch Summary)

Een moeder nam contact op met onze afdeling Medische Genetica omdat haar zoon net als twee broers, een oom en een neef, met een verhemelte spleet is geboren.
Bij het uitvragen van de familieanamnese kwam naar voren dat bij moeder meer dan 6 gebitselementen niet waren aangelegd.
Hierop ingaand, gaf zij aan dat dit voor haar familie niets bijzonders is: " Bij heel veel familieleden zijn een groot aantal tanden en kiezen niet aangelegd. "

Een schisis, dat is een lip-kaakspleet en/of verhemeltespleet, is een veel voorkomende aangeboren afwijking waarmee in Nederland jaarlijks ongeveer 300-350 kinderen worden geboren.
Een schisis gaat vaak gepaard met andere afwijkingen.
Zo is opvallend dat bij patienten met een schisis vaker tanden of kiezen niet zijn aangelegd (agenetisch). Vaker dan men op grond van het voorkomen van het aangeboren ontbreken van gebitselementen (hypodontie) in de normale bevolking zou verwachten.
Het voorkomen van hypodontie bij een schisis loopt in verschillende studies uiteen van 5% tot 77% ten opzichte van 5.5% in de normale populatie. Het voorkomen van ernstige hypodontie, waarbij met uitzondering van de verstandskiezen, 6 of meer tanden of kiezen agenetisch zijn (gedefinieerd als oligodontie), wordt geschat op 0.14% van de kaukasische bevolking.
Hoewel hypodontie bij schisis deels verklaard zou kunnen worden door de schisis zelf of door chirurgisch ingrijpen, blijkt hypodontie ook meer frequent te zijn wanneer bij het vaststellen van het aantal agenetische gebitselementen de schisis-regio niet wordt meegenomen. In deze situatie bedraagt de prevalentie van hypodontie ongeveer 30%.
Schisis en hypodontie zijn beide complexe aandoeningen waarbij in de etiologie vaak genetische en/of omgevingsfactoren een rol spelen. Bij een deel van de patiënten met schisis en hypodontie is sprake van een onderliggende syndromale aandoening. Er zijn ongeveer 50 syndromen beschreven waarbij zowel schisis als hypodontie verschijnselen zijn van eenzelfde genetische aanleg (OMIM website, London Medical Databases (LMD)).
Diagnostisch onderzoek is van groot belang voor de behandeling, zorg en erfelijkheids-voorlichting van patienten met schisis en/of hypodontie en hun familieleden.
In de klinische praktijk blijken de mogelijke genetische factoren bij het merendeel van de patiënten met schisis en hypodontie helaas niet bekend. In de erfelijkheidsvoorlichting

wordt gebruik gemaakt van herhalingsrisico's die dan meer gebaseerd zijn op ervaring, zonder inzicht in de genetische oorzaak.

De verhoogde prevalentie van hypodontie bij schisis veronderstelt een gemeenschappelijke etiologie. Deze heeft zijn basis in de duidelijke anatomische relatie, vergelijkbare termijn van vorming en overeenkomstige morfologische en moleculaire mechanismen tijdens de embryonale ontwikkeling.
In dit proefschrift hebben we onderzoek verricht naar de rol van het *MSX1* gen bij schisis, hypodontie en ook bij hydrocefalie. Dit laatste onderzoek werd gemotiveerd door het voorkomen van hydrocefalie bij een muismodel met een *Msx1* genafwijking.

De inleiding van dit proefschrift (**hoofdstuk 1**) beschrijft de vorming van de lip, verhemelte en gebit tijdens de embryonale ontwikkeling. Hierbij worden ter illustratie een aantal belangrijke moleculaire *pathways* en genen belicht die een rol spelen in zowel de vorming van gelaat en palatum als de tandaanleg. Ook worden enkele hieraan gerelateerde syndromen beschreven.

Schisis gaat vaak gepaard met andere afwijkingen waardoor er sprake kan zijn van een onderliggende chromosomale afwijking.
In **hoofdstuk 2** wordt het voorkomen van bijkomende aangeboren afwijkingen en chromosoomafwijkingen bij verschillende schisis-categorieën (gespleten lip (Cleft Lip, CL); gespleten lip- kaak – en verhemelte (Cleft Lip & Palate, CLP); gespleten verhemelte (Cleft Palate, CP) beschreven.
De resultaten zijn gebaseerd op een systematische review van publicaties vanaf 1995, aangevuld met data die zijn verkregen uit de Nederlandse Vereniging voor Schisis en Craniofaciale Afwijkingen (NVSCA).
Uit het onderzoek komt naar voren dat de prevalentie van geassocieerde afwijkingen duidelijk gerelateerd is aan de schisis-categorie. Een CL blijkt de meest gunstige prognose te hebben. Dit geldt zowel voor kinderen waarbij de schisis tijdens de zwangerschap bij echo-onderzoek is vastgesteld als voor pasgeboren kinderen met een schisis.
Kinderen met een CP hebben het meest frequent bijkomende aangeboren afwijkingen. Een CP wordt echter bij uitzondering tijdens de zwangerschap vastgesteld.
Indien er naast de schisis geassocieerde afwijkingen zijn, is de kans op een chromosoomafwijking duidelijk hoger. Het ontbreken van geassocieerde afwijkingen sluit een chromosoomafwijking echter niet uit. Door de ontwikkeling van nieuwe DNA

technieken, zoals microarray analyse, kunnen submicroscopische chromosomale afwijkingen worden vastgesteld die met conventioneel chromosoomonderzoek niet kunnen worden aangetoond. Ook bij kinderen met een geïsoleerde schisis blijkt bij microarray analyse soms toch sprake van een onderliggende submicroscopische chromosoomafwijking.
Op grond van deze systematische review hebben we een advies voor prenataal onderzoek bij prenataal vastgestelde schisis geformuleerd.

In **hoofdstuk 3** hebben we vastgesteld dat er een relatie bestaat tussen het *MSX1* gen en schisis bij de mens. In dit hoofdstuk presenteren we een familie waarin verschillende familieleden schisis en/of hypodontie hebben. We hebben aangetoond dat in de aangedane familieleden sprake is van een *MSX1* genafwijking, een zogenaamde nonsens mutatie (p.Ser111*) die een vroegtijdige stop van de translatie veroorzaakt. We zijn tot dit kandidaatgen gekomen, gebaseerd op het gegeven dat bij *knockout* muizen, waarbij het *Msx1* gen is uitgeschakeld, eenzelfde combinatie van schisis en hypodontie aanwezig is zoals binnen de beschreven familie. Door onze bevinding is duidelijk geworden dat het *Msx1* gen niet alleen gepaard gaat met schisis in de muis, maar MSX1 ook een rol kan spelen bij schisis bij de mens.

In **hoofdstuk 4** beschrijven we de rol van MSX1 in de embryonale ontwikkeling met name toegespitst op de vorming van het gelaat, verhemelte en gebit. MSX1 is van belang voor een goede regulatie van cel proliferatie, cel differentiatie en apoptose. Dit zijn cruciale celbiologische processen in de embryonale ontwikkeling. De aanwezigheid van schisis en hypodontie bij een MSX1 mutatie kan goed verklaard worden vanuit verstoring van deze processen tijdens de embryonale ontwikkeling.

In **hoofdstuk 5** onderzochten we het verband van *MSX1* en omgevingsfactoren in relatie tot schisis bij het kind. Op basis van een case-control studie (176 schisispatienten en 146 controles) vonden we een toegenomen kans op schisis bij kinderen van rokende vrouwen die zwanger zijn van een kind met een bepaalde *MSX1* allel. Deze kans op schisis blijkt nog meer te zijn toegenomen als beide ouders roken.

In **hoofdstuk 6** wordt het onderzoek beschreven naar het voorkomen van mutaties in het *MSX1* gen en andere bekende kandidaatgenen voor hypodontie (*PAX9*, *IRF6, AXIN2)* binnen een Nederlandse patiëntengroep met geïsoleerde ernstige hypodontie. Op grond van recente inzichten in de rol van WNT10A in de tandontwikkeling en de bevinding dat

patiënten met autosomaal recessieve odonto-onycho-dermale-dysplasie door mutaties in het *WNT10A* gen (OMIM 257980; OODD) vaker hypodontie hebben, postuleerden we dat het *WNT10A* gen een zeer interessant kandidaatgen is voor niet-syndromale hypodontie. We besloten binnen de patiëntenpopulatie met geïsoleerde ernstige hypodontie ook mutatieanalyse van het *WNT10A* gen te verrichten.
Ons onderzoek toonde aan dat mutaties in *WNT10A* een frequente oorzaak zijn van niet-syndromale hypodontie. Door *WNT10A* mee te nemen in de genetische diagnostiek bij niet-syndromale hypodontie wordt de opbrengst sterk verhoogd van 15% naar 71%.
We stellen dan ook vast dat mutaties in het *WNT10A* gen veel vaker een rol spelen bij patiënten met hypodontie dan mutaties in het *MSX1* gen. De verschijnselen bij een *WNT10A* mutatie blijken in ernst wel te variëren.

In **hoofdstuk 7** ligt de nadruk op de tandmorfologie. We wilden nagaan of de tandmorfologie een aanwijzing zou kunnen zijn voor de mogelijk onderliggende genetische oorzaak van de hypodontie. Om meer inzicht te krijgen in het dentale fenotype bij personen met een *MSX1* mutatie, hebben we gebruik gemaakt van een nieuw ontwikkelde 3-D tandmorfologische meettechniek. In totaal zijn acht personen uit de in hoofdstuk 3 beschreven familie onderzocht met deze nieuwe methode. In deze personen, waarbij een nonsens mutatie (p.Ser111*) is vastgesteld in het *MSX1* gen zijn verschillende aspecten van de tandmorfologie bekeken. We hebben vastgesteld dat er significante vormverschillen van de centrale snijtand in de bovenkaak bestaan tussen personen met de *MSX1* mutatie en controlepersonen.
De tandvorm van de patiënten met de *MSX1* mutatie blijkt zich te onderscheiden van patiënten met hypodontie in het algemeen. Bij hypodontie worden vaak kleinere gebitselementen gezien dan in de controlegroep. Een mogelijke verklaring voor de bevinding bij patiënten met een mutatie wordt besproken.
Een beter inzicht in dentale fenotype-genotyperelatie zou kunnen bijdragen in een vroege herkenning van een genetische oorzaak voor de hypodontie en zo genetische diagnostiek richting kunnen geven.

In **hoofdstuk 8** hebben we een kleine serie patiënten met een hydrocefalie op basis van een aquaductstenose getest voor mutaties in het *MSX1* gen.
Zoals in de inleiding beschreven blijken *Msx1 knockout*-muizen, waarbij het *Msx1* gen is uitgeschakeld naast de schisis en hypodontie vaak een hydrocefalie te hebben op basis van een aquaductstenose. Bij de mens is deze relatie niet gerapporteerd.
Bij deze studie werd bij 1 proband een mutatie in het *MSX1* gen vastgesteld. Bij een

familielid die drager is van dezelfde *MSX1* mutatie werden enkele milde morfologische veranderingen op de MRI gezien. Deze bevindingen geven steun aan de hypothese dat *MSX1* betrokken kan zijn in de etiologie van hydrocefalus bij de mens. We realiseren ons dat we slechts een klein aantal patiënten hebben kunnen onderzoeken en stellen dat verder onderzoek naar de mogelijke relatie tussen *MSX1* en hydrocefalie bij de mens nodig is.

In **hoofdstuk 9** worden de bevindingen samengevat en bediscussieerd.
Met het onderzoek is meer inzicht verkregen in de rol van *MSX1* in de etiologie van schisis en hypodontie bij de mens. Geconcludeerd kan worden dat *MSX1* mutaties zeldzaam zijn in patiënten met schisis en/of hypodontie. Mutaties in het *WNT10A* gen verklaren meer dan de helft van de patiënten met hypodontie. Een mogelijke relatie tussen het *MSX1 g*en en hydrocefalie bij de mens wordt ondersteund.

In de discussie van **hoofdstuk 9** wordt ingegaan op het erfelijkheidsonderzoek en –voorlichting bij schisis en hypodontie in de klinische praktijk.
Op dit moment is het niet mogelijk om bij alle patiënten met schisis en hypodontie een genetische oorzaak vast te stellen. Het is zeer aannemelijk dat bij een deel van de patiënten met geïsoleerde hypodontie en schisis nog onbekende 'hypodontiegenen' en 'schisisgenen' een rol spelen. Besproken wordt dat kandidaatgenbenadering en kennis van syndromen, ook vandaag de dag nog behulpzaam kan zijn bij de identificatie van nog onbekende genetische oorzaken. Voorts kan een goede classificatie van de schisis en hypodontie en beschrijving van het volledige fenotype van patiënt en familie een belangrijke bijdrage leveren in het identificeren van nieuwe hypodontie- en schisisgenen. Toepassing van nieuwe DNA-technieken (met name compleet genoomonderzoek door *next generation sequencing*) biedt nu grote mogelijkheden bij het identificeren van deze genen. De interpretatie van de resultaten vormt daarbij een grote uitdaging. Bij de zoektocht van nieuwe genen is kennis van embryologie, ontwikkelingsbiologie en bio-informatica essentieel. Tevens is multidisciplinaire samenwerking in de patiëntenzorg en samenwerking tussen onderzoeksgroepen in binnen- en buitenland van groot belang.

Curriculum Vitae

Marie-José van den Boogaard was born on august 2nd, 1964 in Helmond as one of a twin and the youngest of four children.
In 1983 she passed her secondary school at the Niels Stensen College in Utrecht. In the same year, she started her medical training at the Medical Faculty of the University of Utrecht. During her study she assisted Prof. dr. R.C.M.Hennekam on his study on Rubinstein-Taybi syndrome and did an elective internship at the Clinical Genetic Center Utrecht. These inspiring years strengthened her ambition to become a clinical geneticist.

After obtaining her medical degree in 1990, she started working at the Clinical Genetic Center Utrecht. Subsequently, she started her residency in Clinical Genetics Utrecht under supervision of Prof.dr. F.A. Beemer. In January 1997 she was registered as Clinical Geneticist.
Since then she has been working at the Department of Medical Genetics of the University Medical Center Utrecht (current head: Prof.dr. N.V.A.M. Knoers). In 2000 she reported the identification of *MSX1* as a causative gene for monogenetic orofacial clefting in combination with hypodontia in a Dutch family. This specifically triggered further study on *MSX1* in relation to orofacial clefting, hypodontia and hydrocephaly, resulting in the current thesis (promotor: Prof.D.Lindhout).

From 2005-2010 she was chairman of the LCO (landelijk cursorisch onderwijs) committee of the Dutch Society for Clinical Genetics (Vereniging Klinische Genetica Nederland, VKGN) organizing a national educational program for clinical geneticists in training.

Her clinical interest covers the broad field of dysmorphology, with a focus on orofacial anomalies.

Marie-José van den Boogaard is married with Paul Hendriks. They have three children Bram, Freek and Evy.

Dankwoord

Het dankwoord. Ik ben heel erg blij en dankbaar dat ik dit hoofdstuk nu mag schrijven. Niet alleen omdat ik het, na een hele drukke tijd, misschien iets rustiger ga krijgen. Maar vooral omdat ik mag terugkijken op een tijd waarin we met veel plezier een aantal antwoorden hebben mogen vinden op vragen die we in de kliniek tegenkwamen. Hierbij wil ik uiteraard alle patiënten en familieleden bedanken voor hun vertrouwen, medewerking, en interesse.
Ik moet toegeven dat antwoorden natuurlijk altijd weer nieuwe vragen oproepen. Vragen die mij enthousiast maken om ook hierop het antwoord te willen vinden en meer inzicht te krijgen in de mechanismen die een rol spelen. Antwoorden en inzichten waar patiënten en onderzoekers mee verder kunnen.

Dit enthousiasme heeft zeker te maken met de inspirerende en prettige samenwerking met heel veel mensen die ik in de afgelopen jaren heb mogen ervaren. Zonder deze mensen was dit proefschrift niet tot stand gekomen.
Ik wil al deze mensen hierbij hartelijk danken!

Een aantal mensen wil ik graag in het bijzonder bedanken.

Dr. Ploos van Amstel, beste Hans-Kristian, ik ben echt heel erg blij met jou als co-promotor. Allereerst wil ik je bedanken voor al je tijd en geduld. Als ik weer eens binnenrende, op een voor jou niet altijd even handig moment, liep ik even later altijd weer tevreden de kamer uit. De ene keer omdat je met een relativerende blik alles weer in perspectief bracht, een andere keer door te zorgen dat ideeën gelijk vorm kregen en werden opgestart. Ik ben je ook heel dankbaar dat je altijd enthousiast was over mijn 'nieuwe' ideeën. En dat we daar dan samen mee aan de slag gingen. Hans Kristian, zonder jouw betrokkenheid was dit proefschrift er niet geweest. Heel erg bedankt daarvoor.

Prof. Dr. Lindhout, beste Dick. Ik weet nog goed dat je met het idee kwam van een promotieonderzoek. Dit was in een fase dat Bram, Freek en Evy nog heel klein waren. Zoals je weet had ik mijn twijfels over promoveren. Ik wil je heel erg bedanken voor de ruimte die je me hierin hebt gegeven. Het vertrouwen dat je mij gaf als promotor, in combinatie met je enthousiasme en optimisme waren voor mij precies de goede voedingsbodem voor dit proefschrift. Ik ben je hier heel erg dankbaar voor.

Prof. Dr. Knoers, beste Nine. Toen je als nieuw hoofd bij onze afdeling kwam werken, was ik al een stukje op weg met dit proefschrift. Ik ben heel blij dat je mij de ruimte en tijd hebt gegeven om me te concentreren op het afronden ervan. Door jou is het onderzoek in een stroomversnelling gekomen. Ik waardeer ook in het bijzonder je enthousiasme, je betrokkenheid en je altijd heldere visie. Ik wil je ook bedanken voor jouw openheid en de ruimte die je geeft om dingen met je te bespreken. Ik ben je hier heel dankbaar voor.

Prof. Dr. Steegers-Theunissen, beste Régine. Ik wil jou in het bijzonder noemen en bedanken voor onze samenwerking in het beantwoorden van een van de belangrijke onderzoeksvragen van dit proefschrift en het geduld en vertrouwen dat je hierin had. Ik heb onze samenwerking heel erg gewaardeerd. Heel veel dank hiervoor.

Prof. Dr. Cune, beste Marco. Ik ben heel blij dat jij heel graag meer wilde weten van de genetica en dan met name van de genetica van hypodontie. Jouw enthousiasme genereert energie en maakt gezamenlijk onderzoek doen met jou heel bijzonder. Ik hoop dat we in de toekomst nog heel lang samen onderzoek kunnen doen.

Prof. Vermeij-Keers, beste Christl. Ik wil jou in het bijzonder bedanken voor jouw waardevolle bijdrage aan dit proefschrift en onze hele plezierige samenwerking. Ik ben je ook heel dankbaar voor jouw kritische blik en toelichting op de embryologie. Al in de beginfase van mijn interesse voor het *MSX1* gen, hadden we interessante discussies over de rol van MSX1 in het ontstaan van schisis in relatie tot de verschillende schisis categorieën. Ik heb dit alles zeer gewaardeerd, en kijk uit naar een verdere samenwerking.

Dr. Frits Meijlink, heel veel dank voor het kritisch lezen van het manuscript over de achtergronden van *Msx1* en je waardevolle suggesties.

Dr. Krapels, beste Ingrid. Ik weet nog dat ik aan het worstelen was met mijn data uit het laboratorium, en jij geen moment aarzelde om me hierbij te helpen. Ik wil je hiervoor heel erg bedanken. We hebben onszelf heel veel vragen voorgelegd, en er zijn heel veel mailtjes over en weer gestuurd. Ik weet dat je hart nu bij de cardiogenetica ligt, maar mocht je toch nog iets voelen voor samenwerking in onderzoek naar orofaciale afwijkingen, dan lijkt me het heel leuk om onze samenwerking weer nieuw leven in te blazen.

Dr. Mink van der Molen, beste Aebele. Ik ben heel blij dat je het initiatief hebt genomen voor het schrijven van een richtlijn voor counseling na prenataal vastgestelde schisis. Met jou als voorzitter is er nu een landelijk en multidisciplinair advies geformuleerd. Ik waardeer jouw enthousiasme en de 'drive' om nieuw onderzoek te initiëren in het bijzonder. Ik weet dat je bij mij, gezien mijn drukke bezigheden, vaak geduld moest opbrengen. Heel veel dank voor je begrip en ik ga ervan uit dat dit nu niet meer nodig is.

Wies Maarse en Annemarie Rozendaal. Wat was onze studie een grote klus en wat hebben we er veel tijd in gestopt! Ik ben heel blij dat we samen een heel mooi overzicht hebben kunnen geven van de bijkomende afwijkingen en chromosoomafwijkingen bij schisis. Heel erg bedankt voor onze prettige samenwerking.

Dr. Créton, beste Marijn. Wat fijn dat Marco ons aan elkaar heeft voorgesteld. Het heeft geresulteerd in een voor mij hele bijzondere en enthousiaste samenwerking. Ik waardeer je heel erg in je werk, maar ook als persoon. Het is geweldig om samen al weer plannen te maken voor nieuw gezamenlijk onderzoek.

Dr. Gooskens, beste Rob. Ik wil je bedanken voor de manier waarop je altijd interesse toonde in mijn promotie. Je gaf me op een prettige manier suggesties, waardoor ik gemotiveerd bleef en niet afhaakte.

Dr. Nievelstein, Rutger-Jan, bedankt dat ik altijd bij je terecht kan om even een foto te bespreken. En bedankt voor je hulp en snelle en vlotte antwoorden.

Dr. Benoît Robert. Thanks for providing the nice pictures of Msx1 expression during mouse development published in this thesis. It' s an honour; I could use the results of such a nice work.

Ellen van Binsbergen en Dr. Heleen Schuring-Blom, heel erg bedankt voor jullie tijd en hulp bij het beschrijven van de karyotypen volgens de juiste nomenclatuur en het meedenken! Iedere keer als ik weer langskwam bleven jullie enthousiast.

Eric Hennekam, bedankt voor het genealogisch onderzoek dat je hebt verricht. En vooral bedankt dat je zo snel met resultaten kon komen zodat deze in het artikel konden worden meegenomen. Bedankt hiervoor!

Het is al weer even geleden dat ik op het laboratorium mocht rondlopen. Maar ik weet nog heel goed dat ik als clinicus regelmatig om hulp verlegen zat. Gelukkig was er dan altijd weer een helpende hand die me te hulp schoot. Patrick, Rumó, Richard, Gert-Jan, Wendy, Yvon, Marjon, Irene, Edith, en Bonamy. Heel erg bedankt hiervoor!

Prof. Dr. Beemer, beste Frits. Ik ben ontzettend dankbaar dat ik bij jou in opleiding tot klinisch geneticus kon komen. Niet alleen omdat ik het vak van de klinische genetica, in al zijn facetten, heel erg bijzonder vind en ik heel blij ben dat ik in deze tijd in dit interessante vakgebied mag werken. Maar vooral omdat ik veel van jou mocht en kon leren. Ik heb grote waardering voor je bescheidenheid, ondanks je grote vakkennis, de wijze waarop je deze kennis overbracht en de zeer prettige manier waarop je met mensen omgaat.
Je wist mij op een hele plezierige maar vasthoudende wijze te overtuigen dat ik toch maar eens moest gaan promoveren. Heel erg bedankt!

Prof. Dr. Hennekam, beste Raoul. Ik weet nog heel goed dat ik als student bij jou langs ging om te vragen of ik je misschien kon ondersteunen in je werkzaamheden. Je was meteen enthousiast en voor ik het wist reden we naar verschillende instituten voor je onderzoek naar het Rubinstein-Taybi syndroom en was ik MCPP's aan het maken en dermatoglyfen aan het bestuderen. Het was geweldig om meegenomen te worden in jouw enthousiasme voor het vak. Ik ben dan ook heel blij dat ik het vak van de dysmorfologie, waarvan ik heb ervaren dat het nog steeds heel waardevol is bij wetenschappelijk onderzoek in de klinische genetica, van jou mocht leren. Ik wil je graag ook op deze plek hiervoor bedanken.

Dr. Margreet Ausems, bedankt dat je, achter de schermen, altijd de vinger aan de pols hield en waar nodig met goede suggesties kwam. Vooral ook bedankt voor het meedenken en het mede organiseren van deze dag.

Carla van der Sijs-Bos, bedankt dat je ervoor gezorgd hebt dat patiëntenzorg en onderzoek voor mij beter te combineren viel.

Paulien Terhal. We zijn al heel wat jaartjes kamergenoten en je bent voor mij een hele bijzondere collega en vriendin. Bedankt voor al je opbeurende, relativerende woorden en kaartjes als ik het weer even niet zag zitten. Het maakte echt een verschil. Ik ben dan ook heel blij dat jij mijn paranimf wilt zijn.

Niet het minst wil ik de afdeling klinische genetica en alle fijne collega's allemaal heel erg bedanken. Het combineren van patiëntenzorg en onderzoek is niet altijd eenvoudig. Ik wil jullie allemaal heel erg bedanken voor alle begrip, geduld, hulp, motiverende en opbeurende woorden, overnemen van werkzaamheden en zoveel meer.

Saskia: Jouw motto: ' A good dissertation' is a ' done dissertation', zal me altijd bijblijven. Ik heb nu in ieder geval ' a done dissertation', waar ik heel blij mee ben. Bedankt voor de gezellige samenwerking. Ik wil jou en je gezin heel veel succes en geluk wensen met je nieuwe start in het zuiden.

Lieve familie en vrienden. De afgelopen tijd hebben we helaas niet zo vaak kunnen afspreken als gewild. Ik wil jullie heel erg bedanken voor jullie begrip en dat jullie altijd met mij mee hebben geleefd. We spreken gauw weer eens af!

Wil en Chris, we kennen elkaar nu al heel wat jaren. Paul en ik zijn heel erg blij dat jullie altijd voor ons klaar staan. Zonder goede zorg voor Bram, Freek en Evy had ik me niet zo op het werk kunnen en willen storten. Het was echt nooit een probleem als ze toch een extra dagje kwamen. En jij, Wil, zorgde er dan ook nog voor dat ik echt niet gestoord werd. En zoals je weet zijn de kinderen ook blij met jou, want ' jij kookt écht veel lekkerder dan papa en mama' . En Chris, bedankt voor al je hulp en voor je aanmoedigende woorden: 'het wordt nu wel eens tijd voor dat feestje'.

Lieve mama, ik wil jou en papa heel erg bedanken voor al jullie liefde en vertrouwen in mij. Ik weet zeker dat papa super trots geweest zou zijn!

Tante Tinie, ik wil u heel erg bedanken voor alle steun. Dat u zo meeleefde bij het tot stand komen van dit proefschrift was voor mij heel waardevol.

An en Theo. Theo, Agnes, Anja en Oebele. Ik realiseer me dat ik de laatste tijd veel te weinig tijd heb gehad voor gezelligheid met de familie. Maar gelukkig komt er met de afronding van dit proefschrift wat meer tijd.

Marion, mijn grote zus en viltmaatje. Gelukkig waren er onze viltavondjes, die ik echt niet kon/mocht afzeggen. Als je heel druk bent, is ontspanning des te belangrijker! Maar ik weet zeker dat ik zonder jou deze ontspannende avondjes had laten schieten. Bedankt voor je gezelligheid.

Michel, ik ben heel blij met jou als broer. Door ons drukke bestaan zien we elkaar minder dan we zouden willen. Ik ben echt heel blij dat jij, ondanks je drukke werk, toch tijd vrij wilde maken voor de vormgeving van dit boekje. En dat allemaal onder een grote tijdsdruk. Dit is echt ons boekje geworden. Super bedankt!

Marie-Louise, ik heb het altijd heel fijn gevonden een tweelingzus te hebben, maar dat jij ook aan mij zijde staat als paranimf is helemaal bijzonder.

Lieve Bram, Freek en Evy. Ik zat soms wel heel erg veel achter de computer en ik kon me heel goed voorstellen dat jullie het liedje “mama, is een stresskip” (van kinderen voor kinderen) uit volle borst meezongen. Jullie zijn geweldig. Papa en ik zijn reuzetrots op jullie! ‘ Ik hou meer van jullie dan van hier naar de maan en terug, en dan nog eens heen en terug, en dan nog eens en nog eens en nog eens….’ Zóveel! En dan nog héél veel meer!

Lieve, lieve Paul. Jij hebt me altijd gesteund en gemotiveerd in mijn werk! Als ik het even niet zag zitten had je een goed advies, nam je de kinderen mee zodat ik verder kon, als tabellen niet lukte hielp je me uit de brand! Dank je wel voor alles!

List of Publications

A systematic review of associated structural and chromosomal defects in oral clefts: when is prenatal genetic analysis indicated?
Maarse W, Rozendaal AM, Pajkrt E, Vermeij-Keers C, Mink van der Molen AB, *van den Boogaard MJ*.
J Med Genet. 2012;49:490-8.

X-exome sequencing identifies a HDAC8 variant in a large pedigree with X-linked intellectual disability, truncal obesity, gynaecomastia, hypogonadism and unusual face.
Harakalova M, *van den Boogaard MJ*, Sinke R, van Lieshout S, van Tuil MC, Duran K, Renkens I, Terhal PA, de Kovel C, Nijman IJ, van Haelst M, Knoers NV, van Haaften G, Kloosterman W, Hennekam RC, Cuppen E, Ploos van Amstel HK.
J Med Genet. 2012;49:539-43.

Three-dimensional analysis of tooth dimensions in the MSX1-missense mutation.
Créton M, *van den Boogaard MJ*, Maal T, Verhamme L, Fennis W, Carels C, Kuijpers-Jagtman AM, Cune M.
Clin Oral Investig. 2012 Aug 31.

Mutations in WNT10A are present in more than half of isolated hypodontia cases.
van den Boogaard MJ, Créton M, Bronkhorst Y, van der Hout A, Hennekam E, Lindhout D, Cune M, Ploos van Amstel HK.
J Med Genet. 2012;49:327-31.

Delayed diagnosis and underreporting of congenital anomalies associated with oral clefts in the Netherlands: a national validation study.
Rozendaal AM, Luijsterburg AJ, Ongkosuwito EM, *van den Boogaard MJ*, de Vries E, Hovius SE, Vermeij-Keers C.
J Plast Reconstr Aesthet Surg. 2012;65:780-90.

Heterozygous missense mutations in SMARCA2 cause Nicolaides-Baraitser syndrome.
Van Houdt JK, Nowakowska BA, Sousa SB, van Schaik BD, Seuntjens E, Avonce N, Sifrim A, Abdul-Rahman OA, *van den Boogaard MJ*, Bottani A, Castori M, Cormier-Daire V, Deardorff MA, Filges I, Fryer A, Fryns JP, Gana S, Garavelli L, Gillessen-Kaesbach G, Hall BD, Horn D, Huylebroeck D, Klapecki J, Krajewska-Walasek M, Kuechler A, Lines MA, Maas S, Macdermot KD, McKee S, Magee A, de Man SA, Moreau Y, Morice-Picard F, Obersztyn E, Pilch J, Rosser E, Shannon N, Stolte-Dijkstra I, Van Dijck P, Vilain C, Vogels A, Wakeling E, Wieczorek D, Wilson L, Zuffardi O, van Kampen AH, Devriendt K, Hennekam R, Vermeesch JR.
Nat Genet. 2012;44:445-9

Prenatal ultrasound screening for orofacial clefts.
Maarse W, Pistorius LR, Van Eeten WK, Breugem CC, Kon M, *van den Boogaard MJ*, Mink van Der Molen AB.
Ultrasound Obstet Gynecol. 2011;38:434-9.

MLL2 mutation spectrum in 45 patients with Kabuki syndrome.
Paulussen AD, Stegmann AP, Blok MJ, Tserpelis D, Posma-Velter C, Detisch Y, Smeets EE, Wagemans A, Schrander JJ, *van den Boogaard MJ*, van der Smagt J, van Haeringen A, Stolte-Dijkstra I, Kerstjens-Frederikse WS, Mancini GM, Wessels MW, Hennekam RC, Vreeburg M, Geraedts J, de Ravel T, Fryns JP, Smeets HJ, Devriendt K, Schrander-Stumpel CT.
Hum Mutat. 2011;32:E2018-25.

Copy number changes of the microcephalin 1 gene (MCPH1) in patients with autism spectrum disorders.
Ozgen HM, van Daalen E, Bolton PF, Maloney VK, Huang S, Cresswell L, *van den Boogaard MJ*, Eleveld MJ, van 't Slot R, Hochstenbach R, Beemer FA, Barrow M, Barber JC, Poot M.
Clin Genet. 2009;76:348-56.

Clinical spectrum of SIX3-associated mutations in holoprosencephaly: correlation between genotype, phenotype and function.
Lacbawan F, Solomon BD, Roessler E, El-Jaick K, Domené S, Vélez JI, Zhou N, Hadley D, Balog JZ, Long R, Fryer A, Smith W, Omar S, McLean SD, Clarkson K, Lichty A, Clegg NJ, Delgado MR, Levey E, Stashinko E, Potocki L, Vanallen MI, Clayton-Smith J, Donnai D, Bianchi DW, Juliusson PB, Njølstad PR, Brunner HG, Carey JC, Hehr U, Müsebeck J, Wieacker PF, Postra A, Hennekam RC, *van den Boogaard MJ*, van Haeringen A, Paulussen A, Herbergs J, Schrander-Stumpel CT, Janecke AR, Chitayat D, Hahn J, McDonald-McGinn DM, Zackai EH, Dobyns WB, Muenke M.
J Med Genet. 2009;46:389-98.

The MSX1 allele 4 homozygous child exposed to smoking at periconception is most sensitive in developing nonsyndromic orofacial clefts.
van den Boogaard MJ, de Costa D, Krapels IP, Liu F, van Duijn C, Sinke RJ, Lindhout D, Steegers-Theunissen RP.
Hum Genet. 2008;124:525-34.

Ectrodactyly with fibular aplasia: a separate entity?
Menke LA, Bijlsma EK, van Essen AJ, *van den Boogaard MJ*, van Rijn RR, Cobben JM.
Eur J Med Genet. 2008;51:488-96.

Hypomethylation of the H19 gene causes not only Silver-Russell syndrome (SRS) but also isolated asymmetry or an SRS-like phenotype.
Bliek J, Terhal P, *van den Boogaard MJ*, Maas S, Hamel B, Salieb-Beugelaar G, Simon M, Letteboer T, van der Smagt J, Kroes H, Mannens M.
Am J Hum Genet. 2006;78:604-14.

LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development.
Gong Y, Slee RB, Fukai N, Rawadi G, Roman-Roman S, Reginato AM, Wang H, Cundy T, Glorieux FH, Lev D, Zacharin M, Oexle K, Marcelino J, Suwairi W, Heeger S, Sabatakos G, Apte S, Adkins WN, Allgrove J, Arslan-Kirchner M, Batch JA, Beighton P, Black GC, Boles RG, Boon LM, Borrone C, Brunner HG, Carle GF, Dallapiccola B, De Paepe A, Floege B, Halfhide ML, Hall B, Hennekam RC, Hirose T, Jans A, Jüppner H, Kim CA, Keppler-Noreuil K, Kohlschuetter A, LaCombe D, Lambert M, Lemyre E, Letteboer T, Peltonen L, Ramesar RS, Romanengo M, Somer H, Steichen-Gersdorf E, Steinmann B, Sullivan B, Superti-Furga A, Swoboda W, *van den Boogaard MJ*, Van Hul W, Vikkula M, Votruba M, Zabel B, Garcia T, Baron R, Olsen BR, Warman ML. Osteoporosis-Pseudoglioma Syndrome Collaborative Group.
Cell. 2001 Nov 16;107(4):513-23.

MSX1 mutation is associated with orofacial clefting and tooth agenesis in humans.
van den Boogaard MJ, Dorland M, Beemer FA, Ploos van Amstel HK.
Nat Genet. 2000;24:342-3.

Osteoporosis-pseudoglioma syndrome, a disorder affecting skeletal strength and vision, is assigned to chromosome region 11q12-13.
Gong Y, Vikkula M, Boon L, Liu J, Beighton P, Ramesar R, Peltonen L, Somer H, Hirose T, Dallapiccola B, De Paepe A, Swoboda W, Zabel B, Superti-Furga A, Steinmann B, Brunner HG, Jans A, Boles RG, Adkins W, *van den Boogaard MJ*, Olsen BR, Warman ML.
Am J Hum Genet. 1996 Jul;59(1):146-51.

Glycogen storage disease type II: frequency of three common mutant alleles and their associated clinical phenotypes studied in 121 patients.
Kroos MA, Van der Kraan M, Van Diggelen OP, Kleijer WJ, Reuser AJ, *Van den Boogaard MJ*, Ausems MG, Ploos van Amstel HK, Poenaru L, Nicolino M, et al.
J Med Genet. 1995;32:836-7.

Genotype-phenotype correlation in adult-onset acid maltase deficiency.
Wokke JH, Ausems MG, *van den Boogaard MJ*, Ippel EF, van Diggelene O, Kroos MA, Boer M, Jennekens FG, Reuser AJ, Ploos van Amstel HK.
Ann Neurol. 1995;38:450-4.

The Peters'-Plus syndrome: description of 16 patients and review of the literature.
Hennekam RC, Van Schooneveld MJ, Ardinger HH, *Van Den Boogaard MJ*, Friedburg D, Rudnik-Schoneborn S, Seguin JH, Weatherstone KB, Wittebol-Post D, Meinecke P.
Clin Dysmorphol. 1993;2:283-300.

Identification of two new nucleotide mutations (HPRTUtrecht and HPRTMadrid) in exon 3 of the human hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene.
Bouwens-Rombouts AG, *van den Boogaard MJ*, Puig JG, Mateos FA, Hennekam RC, Tilanus MG.
Hum Genet. 1993;91:451-4.

Deletion at chromosome 16p13.3 as a cause of Rubinstein-Taybi syndrome: clinical aspects.
Hennekam RC, Tilanus M, Hamel BC, Voshart-van Heeren H, Mariman EC, van Beersum SE, *van den Boogaard MJ*, Breuning MH.
Am J Hum Genet. 1993;52:255-62.

Rubinstein-Taybi syndrome caused by submicroscopic deletions within 16p13.3.
Breuning MH, Dauwerse HG, Fugazza G, Saris JJ, Spruit L, Wijnen H, Tommerup N, van der Hagen CB, Imaizumi K, Kuroki Y, *van den Boogaard MJ*, de Pater JM, Mariman EC, Hamel BC, Himmelbauer H, Frischauf AM, Stallings R, Beverstock GC, van Ommen GJ, Hennekam RC.
Am J Hum Genet. 1993;52:249-54.

A case with laryngeal atresia and partial trisomy 9 due to maternal 9;16 translocation.
Van den Boogaard MJ, De Pater J, Hennekam RC.
Genet Couns. 1991;2:83-91.

A cephalometric study in Rubinstein-Taybi syndrome.
Hennekam RC, *Van den Boogaard MJ*, Van Doorne JM.
J Craniofac Genet Dev Biol. 1991;11:33-40.

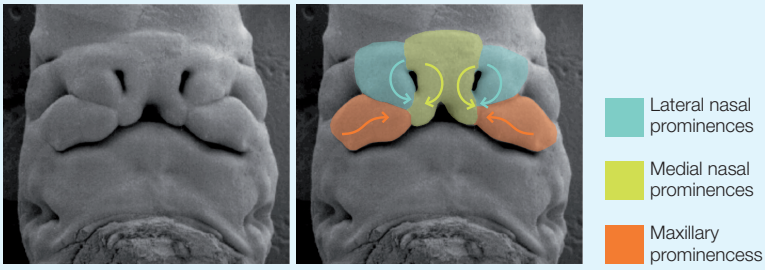
Autosomal dominant craniosynostosis of the sutura metopica.
Hennekam RC, *Van den Boogaard MJ.*
Clin Genet. 1990 Nov;38:374-7.

Metacarpophalangeal pattern profile analysis in Rubinstein-Taybi syndrome.
Hennekam RC, *Van Den Boogaard MJ*, Dijkstra PF, Van de Kamp JJ.
Am J Med Genet Suppl. 1990;6:48-50.

Rubinstein-Taybi syndrome in The Netherlands.
Hennekam RC, *Van Den Boogaard MJ*, Sibbles BJ, Van Spijker HG.
Am J Med Genet Suppl. 1990;6:17-29.

Addenda

Figure 1.1 A.



Human embryo, approximate human age 6 weeks

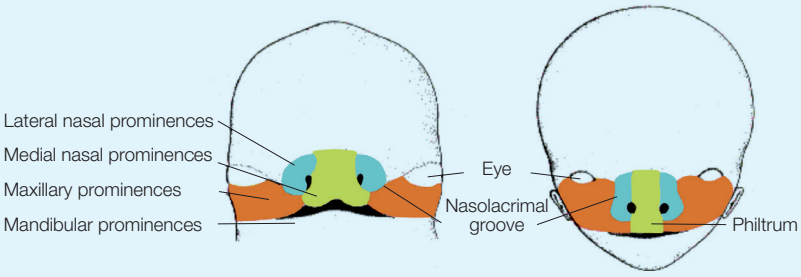


Figure 1.1 A. Embryological lip development | by Drs. Kathleen K. Sulik and Peter R. Bream Jr. with the assistance of Mr. Tim Poe and Ms. Kiran Bindra. https://syllabus.med.unc.edu/courseware/embryo_images/unit-hednk/hednk_htms/hednk031.htm and https://syllabus.med.unc.edu/courseware/embryo_images/unit-hednk/hednk_htms/hednk032.htm. Adapted with permission.

Figure 1.1 B.

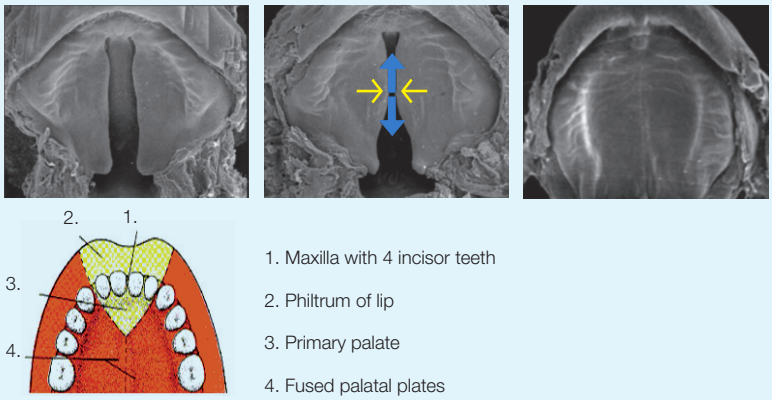


Figure 1.1 B. Embryologic palatal development | by Drs. Kathleen K. Sulik and Peter R. Bream Jr. with the assistance of Mr. Tim Poe and Ms. Kiran Bindra. Human embryo : approximate human age 9 – 10 weeks | https://syllabus.med.unc.edu/courseware/embryo_images/unit-hednk/hednk_htms/hednk039.htm and https://syllabus.med.unc.edu/courseware/embryo_images/unit-hednk/hednk_htms/hednk040.htm - Adapted with permission.

Figure 1.2

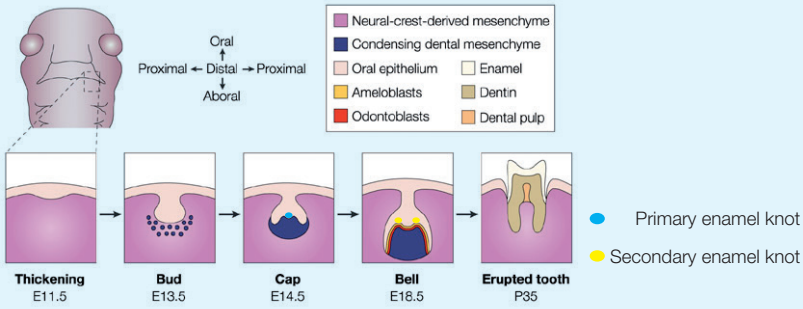


Figure 1.2 Tooth development in the mouse embryo | A schematic frontal view of an embryo head at embryonic day (E)11.5 is shown with a dashed box to indicate the site where the lower (mandibular) molars will form. Below, the stages of tooth development are laid out from the first signs of thickening at E11.5 to eruption of the tooth at around 5 weeks after birth. The tooth germ is formed from the oral epithelium and neural-crest-derived mesenchyme. At the bell stage of development, the ameloblasts and odontoblasts form in adjacent layers at the site of interaction between the epithelium and mesenchyme. These layers produce the enamel and dentin of the fully formed tooth. | By Tucker A, Sharpe P. The cutting-edge of mammalian development; how the embryo makes teeth. Nat Rev Genet. 2004 Jul;5(7):499-508. Adapted with permission by Nature Publishing Group.

Figure 2.1

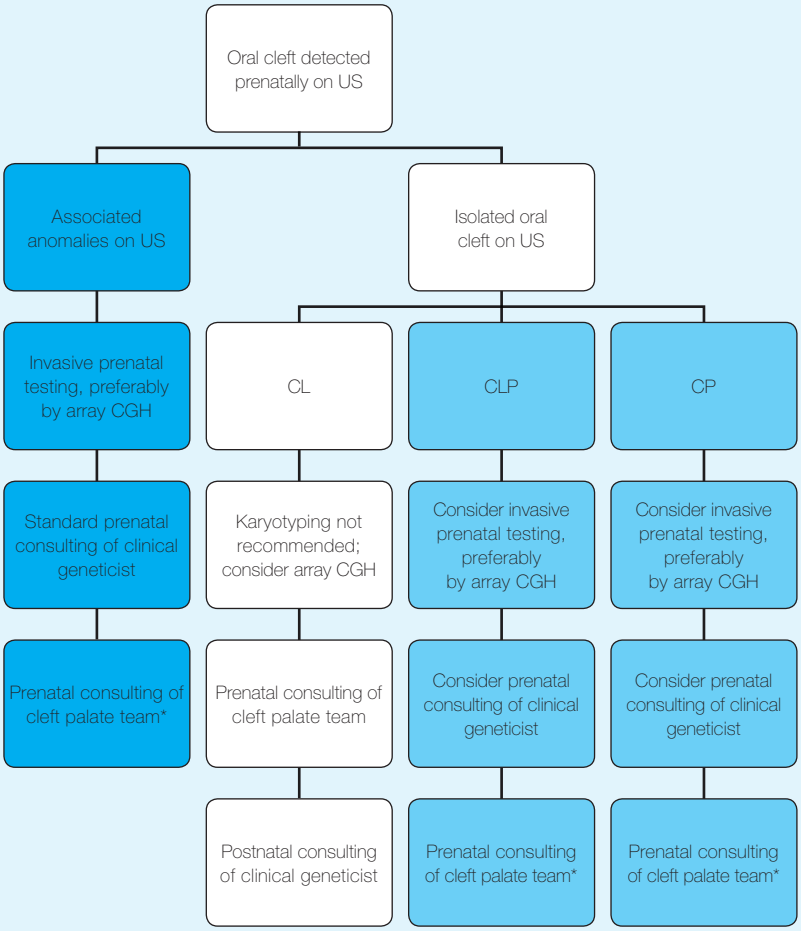
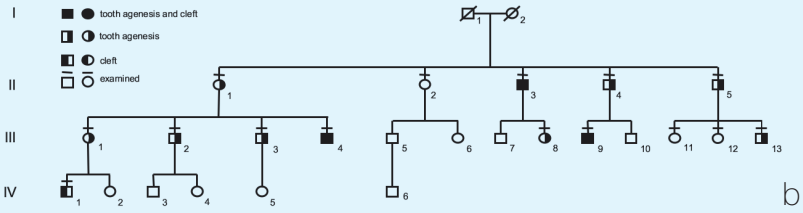


Figure 2.1 Algorithm for invasive genetic testing according to oral cleft category. array CGH, array comparative genomic hybridisation; CL, cleft lip only; CLP, cleft lip with cleft palate; CP, cleft palate only; US, ultrasound. *If a normal karyotype is confirmed or invasive genetic testing is declined.

Figure 3.1



b

Pedigree numbers (fig. 1a)		Left								Right							
		8	7	6	5	4	3	2	1	1	2	3	4	5	6	7	8
II-1	Mx.														•		
	Mn.			•					•	•							
II-4	Mx.	•			•									•			•
	Mn.	•			•									•			•
II-5	Mx.	•			•	•								•			•
	Mn.	•			•									•		•	•
III-1	Mx.	•			•	•							•	•			•
	Mn.	•			•									•			•
III-4	Mx.				•									•			
	Mn.				•									•			
III-8	Mx.				•	•		•			•		•	•			
	Mn.																
III-9	Mx.				•	•		•					•	•			
	Mn.	•			•									•			•
III-13	Mx.	•	•		•			•			•			•		•	•
	Mn.	•	•		•								•	•	•	•	•

b

Figure 3.1 Segregation of clefting and tooth agenesis. | a, Pedigree and symptoms of the Dutch family with clefting and tooth agenesis. | b, Confirmed dentition in affected family members (1, central incisor; 2, lateral incisor; 3, canine; 4, 5, first and second premolar; 6, 7, 8, first, second and third molar, respectively). Mx., maxilla; Mn., mandibula; • , missing teeth.

Figure 3.2

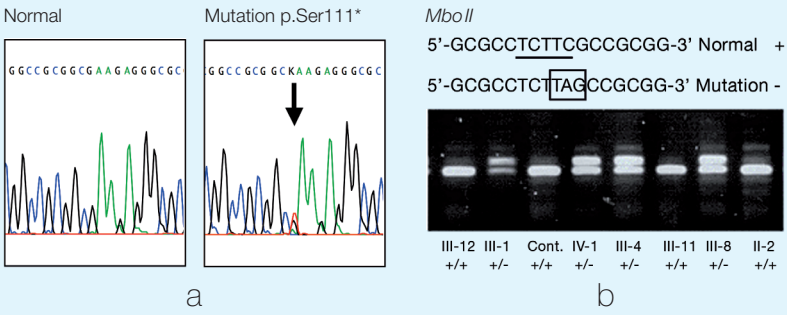


Figure 3.2 *MSX1* nonsense mutation. a. Reverse sequence of a part of exon 1 of *MSX1* showing the substitution producing the p.Ser111*. b. *MboII* digestion after PCR amplification with forward and reverse primers specific for exon 1 (ref. 12). The mutation causes loss of an *MboII* site, resulting in separation of a larger band following agarose electrophoresis.

Figure 4.1

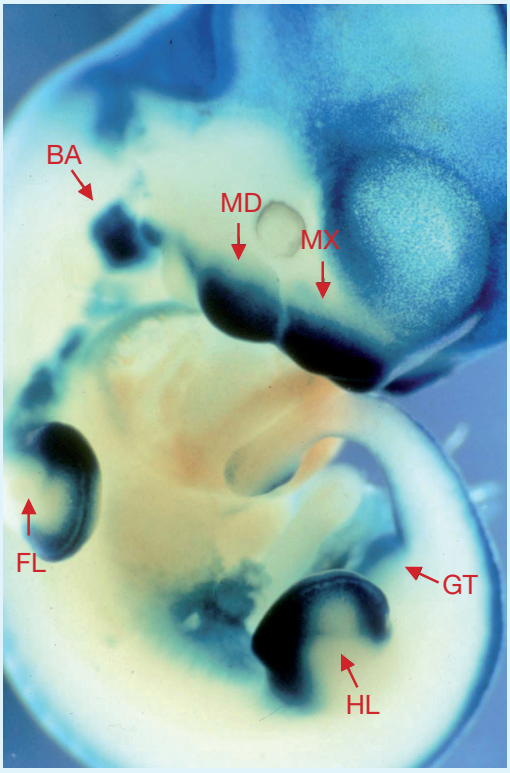


Figure 4.1 Whole-mount heterozygous *Msx1*^{lacZ} mouse embryo stained for b-galactosidase. Stage 11.5 days postcoitum. BA, branchial arch; MD, mandibular process; MX, maxillary process; FL, forelimb bud; HL, hindlimb bud; GT, genital tubercle. (With thanks to D. Houzelstein and B. Robert.)

Figure 4.2

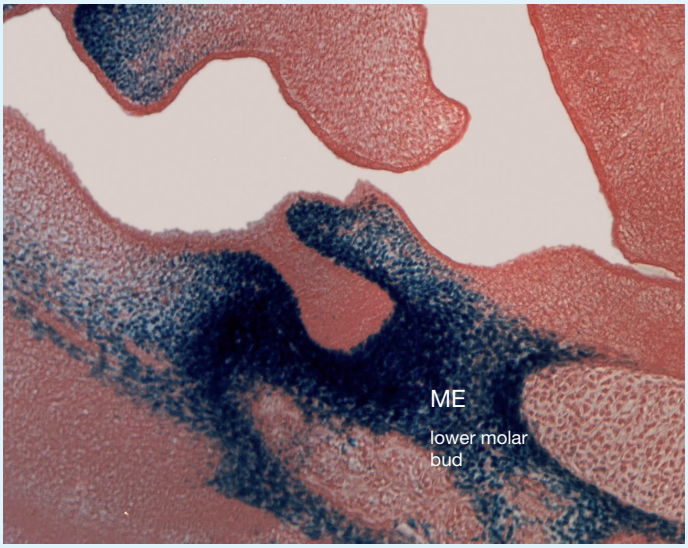


Figure 4.2 *Msx1*^{lacZ} heterozygous mouse embryo stained for b-galactosidase. Stage 13.5 days postcoitum. Magnification of the lower tooth bud. ME, mesectoderm. (With thanks to D. Houzelstein, A. Bach, and B. Robert.)

Figure 4.3

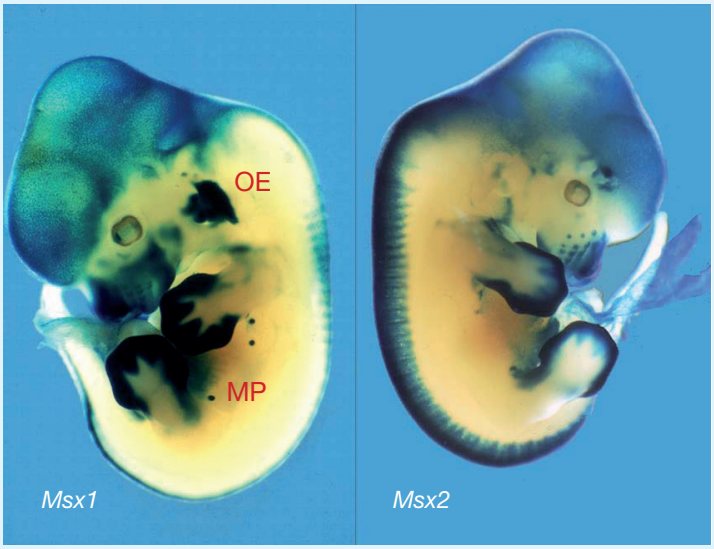


Figure 4.3 Whole-mount heterozygous mouse embryos for *Msx1*^{lacZ} and *Msx2*^{lacZ} stained for b-galactosidase. Stage 12.5 days postcoitum. OE, outer ear; MP, mammary primordium. (With thanks to M.A. Nicola, A. Bach, and B. Robert.)

Figure 4.4

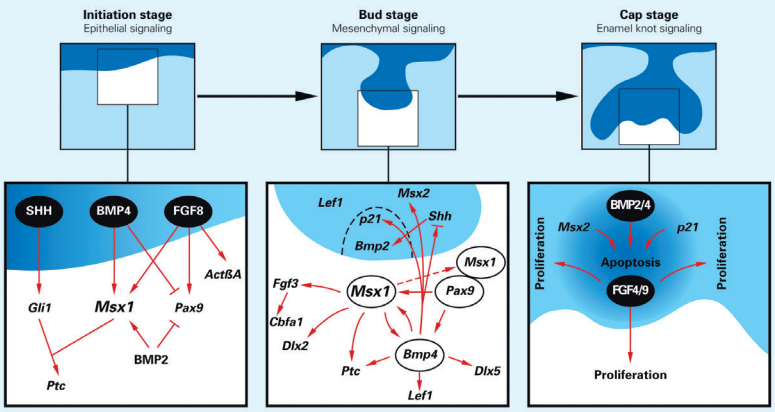


Figure 4.4 Schematic representation of reciprocal signaling between the epithelium and mesenchyme during the early phases of tooth development; initiation stage, bud stage, and cap stage. BMP4, bone morphogenetic protein 4; FGF8, fibroblast growth factor 8; SHH, sonic Hedgehog. (Adapted from Peters and Balling, 1999, and Cohen, 2000, with additional data from Zhang et al., 1999, 2000, Zhao et al., 2000; Yamashiro et al 2003; Zhang et al., 2003a; Ogawa et al., 2006).

Figure 4.5

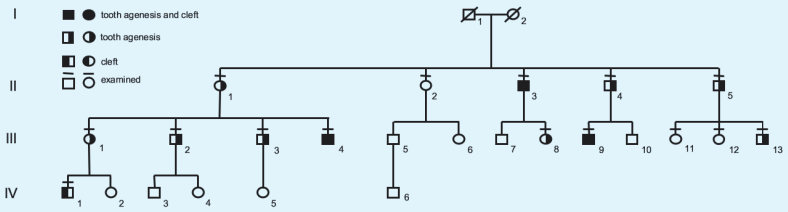


Figure 4.5 Pedigree and symptoms of the Dutch family with the Ser111* *MSX1* mutation.

Figure 4.6

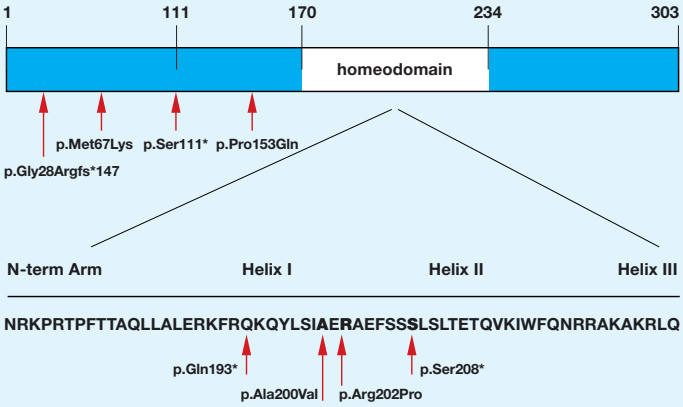


Figure 4.6 Simple schematic diagram of *MSX1* showing the published autosomal dominant *MSX1* mutations in families with tooth agenesis and/or orofacial clefting.

Figure 5.1

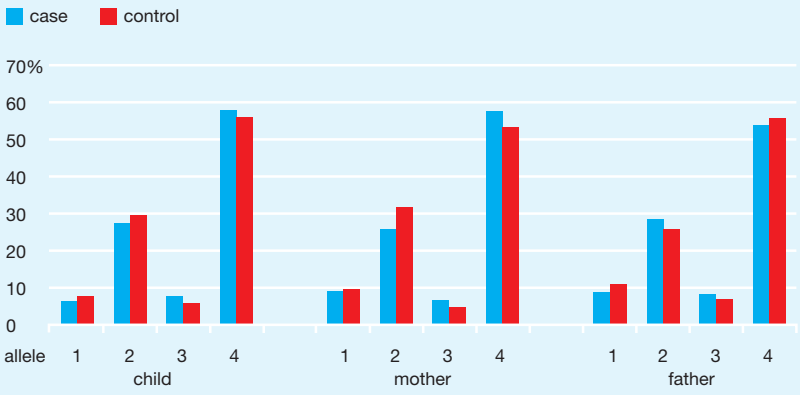


Figure 5.1 Allele frequencies (%) in case and control child, mother, and father.

Figure 6.1

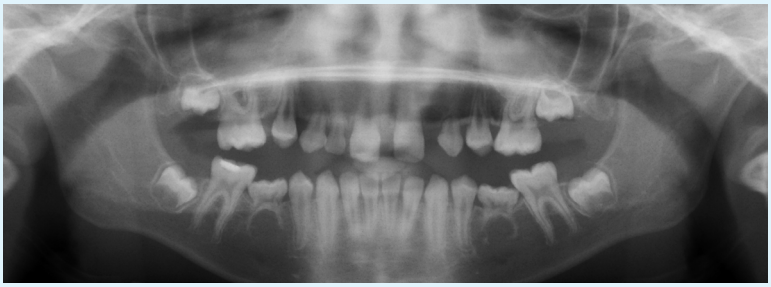


Figure 6.1 Panoramic radiograph of a non-syndromic *WNT10A* hypodontia patient (patient 4; table 6.1).

Figure 7.1

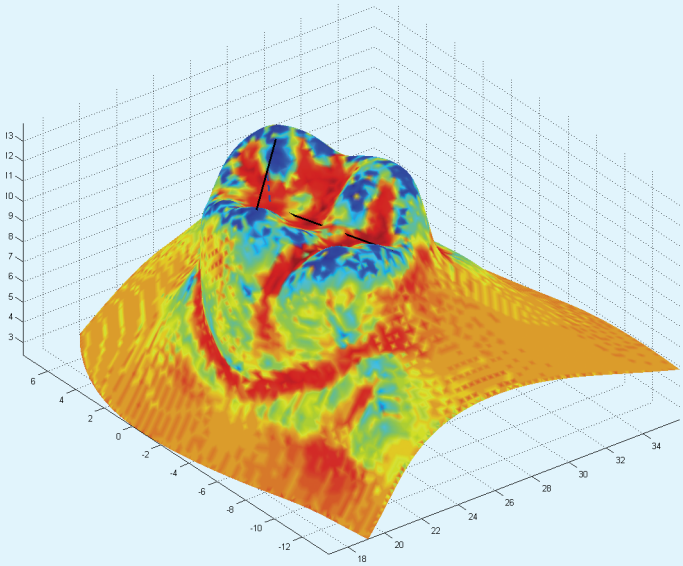


Figure 7.1 The teeth were loaded into a computer program that enables mathematical analysis of three-dimensional shapes. For the first molar, a line is drawn between the two mesial cusp tips. A second line is drawn from the mesial to the distal margin. These two lines are then projected onto each other to form a plane parallel to the occlusal plane.

Figure 7.12

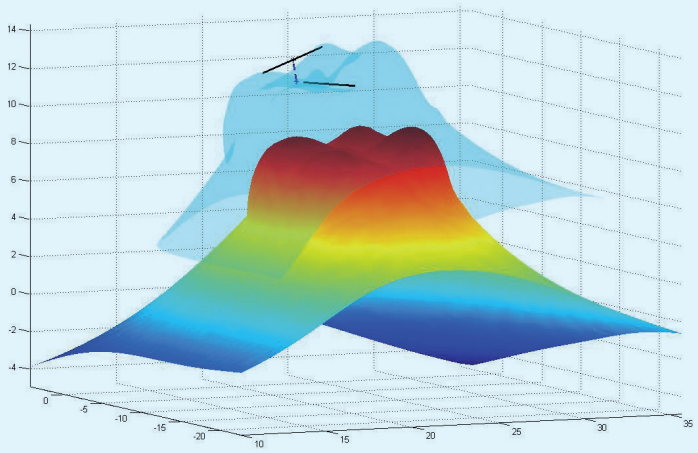


Figure 7.2 The molar is rotated automatically until the constructed plane is parallel to the *X-Y* plane.

Figure 7.3

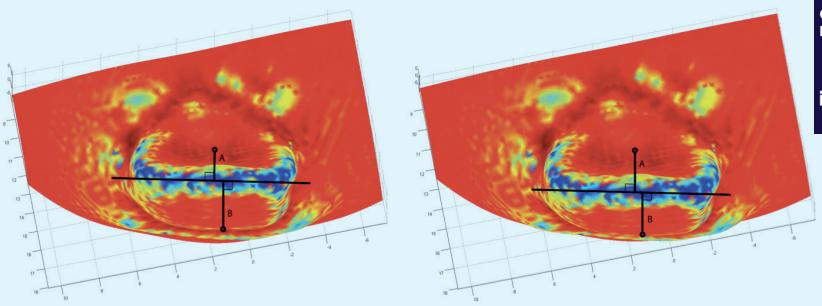


Figure 7.3 To create a plane, the model is rotated along the incisal edge line until the two newly constructed lines (A and B) are equal in length. Line A is drawn perpendicular to the incisal edge line, through the center of the cingulum. Line B is also drawn perpendicular to the incisal edge line, through the most prominent buccal point.

Figure 7.4

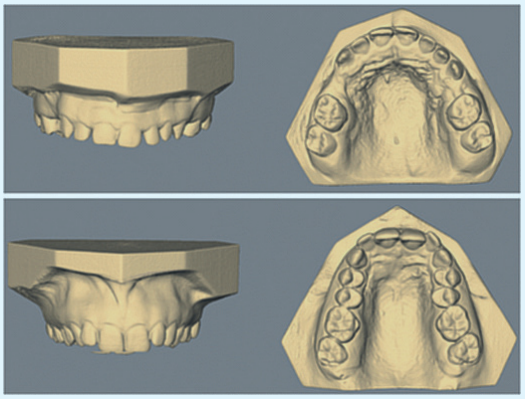


Figure 7.4 a Digital model of the frontal (left) and occlusal (right) views of a typical *MSX1* case (female). It is noticeable that the teeth of the *MSX1* female are distinctive in shape and size compared to a control (i.e., b). In particular, the central incisor has a distinctive "square" appearance. b Digital model of the frontal (left) and occlusal (right) views of a healthy control (female).

Figure 8.1

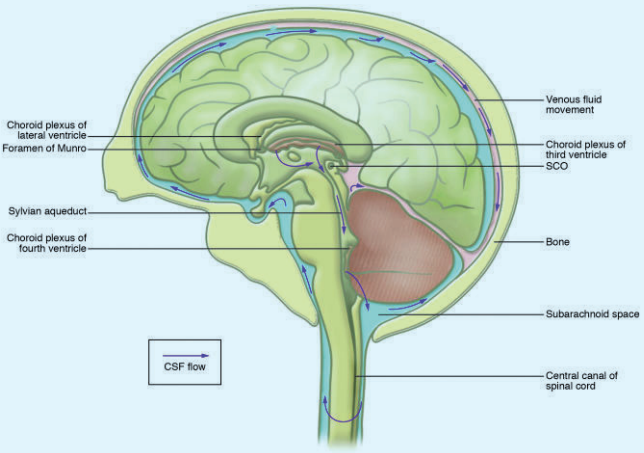


Figure 8.1 CSF flow and the SCO | CSF is secreted from the choroid plexus and travels through the ventricular system in a rostrocaudal direction from the lateral ventricles to the third ventricle via the foramen of Munro, then through the Sylvian aqueduct to the fourth ventricle, and finally into the cisterna magna of the subarachnoid space and the central canal of the spinal cord. CSF fluid is removed through the arachnoid villi into the venous circulation. Adapted from Picketts et al. (2006) – by permission from American Society for Clinical Investigation.

Figure 8.2

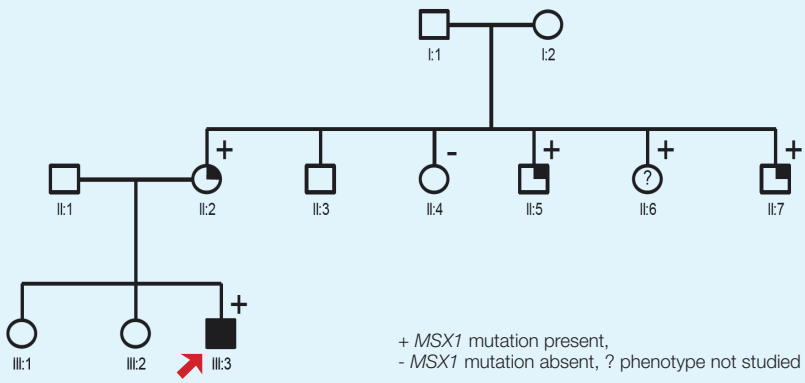


Figure 8.2 Pedigree of patient with hydrocephaly and *MSX1* mutation.

Figure 8.3

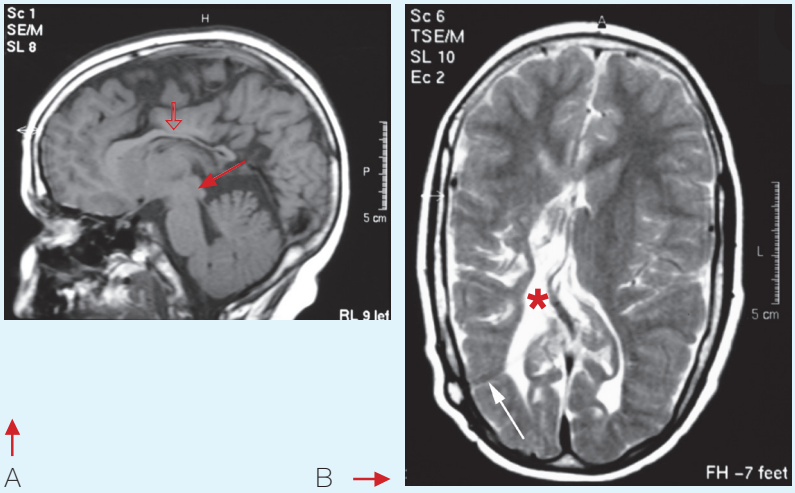


Figure 8.3 MRI of the brain in proband III3, figure 8.2. A. Midsagittal T1 Spin Echo (SE) image illustrating the irregular thinning of the corpus callosum (open arrow) as well as the obliteration of the lumen of the aqueduct (arrow). B. Axial T2 SE image after ventriculo-peritoneal drainage illustrating the somewhat irregular shaped ventricular system, with more expansion of the right lateral ventricle than the left (asterisk), and a diminished amount of white matter. The VP drain is present in the right occipital horn of the lateral ventricle (arrow).

Figure 8.4

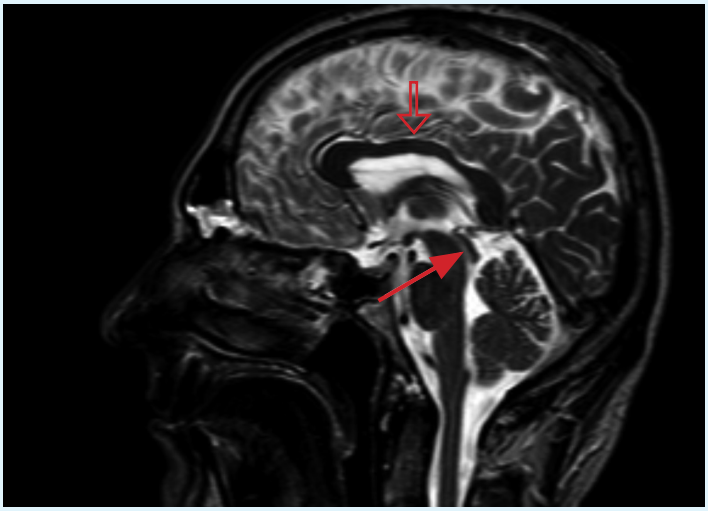
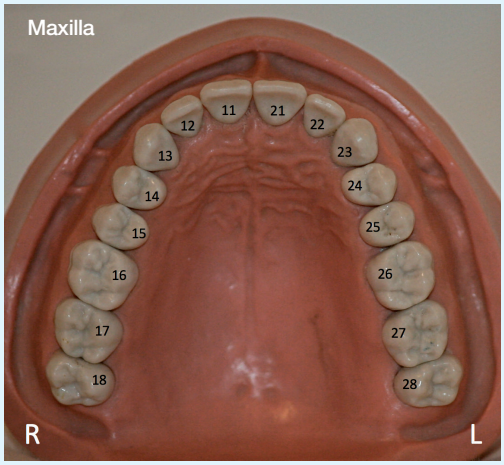
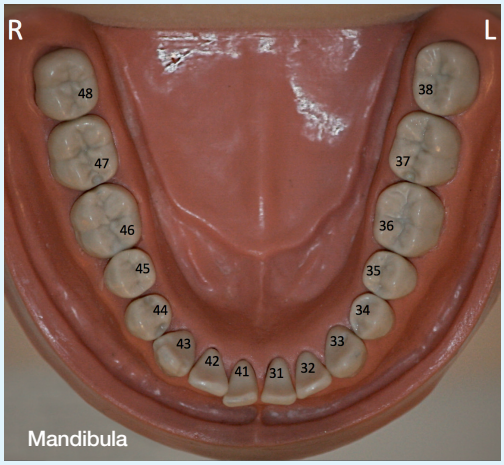


Figure 8.4 MRI of the brain in the uncle of th proband (II7, figure 8.2). The midsagittal T2 Turbo Spin Echo (TSE) image shows the slightly irregular thinning of the truncus of the corpus callosum (open arrow). An aqueductal flow void is lacking (arrow). Flow measurements through the aqueduct did reveal diminished flow through the aqueduct (not shown).

Figure Tooth numbering





Upper teeth

Right	Left
11 central incisor	21 central incisor
12 lateral incisor	22 lateral incisor
13 cuspid (canine)	23 cuspid (canine)
14 first premolar	24 first premolar
15 second premolar	25 second premolar
16 first molar	26 first molar
17 second molar	27 second molar
18 third molar	28 third molar

Lower teeth

Right	Left
41 central incisor	31 central incisor
42 lateral incisor	32 lateral incisor
43 cuspid (canine)	33 cuspid (canine)
44 first premolar	34 first premolar
45 second premolar	35 second premolar
46 first molar	36 first molar
47 second molar	37 second molar
48 third molar	38 third molar

Figure Tooth numbering With thanks to Marijn Créton.

Promotor
Prof. dr. D. Lindhout

Co-promotor
Dr. J.K. Ploos van Amstel

Manuscriptcommissie
Prof.dr. R. Koole
Prof.dr. M.S. Cune
Prof.dr. R.C.M. Hennekam
Prof.dr.ir. J.H.L.M. van Bokhoven

Paranimfen
M-L.E. van den Boogaard
P.A.Terhal