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TRIIN JAGOMÄGI

A study of the genetic etiology of nonsyndromic cleft lip and palate

TARTU UNIVERSITY PRESS

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Dissertation is accepted for the commencement of the degree of Doctor of Philosophy (Medicine) on November 16, 2011 by the Council of the Faculty of Medicine, University of Tartu, Tartu, Estonia.

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The publication of this dissertation is granted by the University of Tartu and by the European union through the European Social Fund.

This research was supported by the Estonian Science Foundation grant ETF7076 and by the European Union through the European Regional Development Fund, in the frame of Centre of Excellence in Genomics and grant no. 3.2.0304.11–0312, Targeted Financing from Estonian Government No. SF0180142s08.

ISSN 1024–395x
ISBN 978–9949–19–931–0 (trükis)
ISBN 978–9949–19–932–7 (PDF)

Tartu Ülikooli Kirjastus
www.tyk.ee
Tellimus nr. 835

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LIST OF ORIGINAL PUBLICATIONS

I. **Jagomägi T**, Soots M, Saag M. Epidemiologic factors causing cleft lip and palate and their regularities of occurrence in Estonia. *Stomatologija* 2010; 12: 105–108.

II. **Jagomägi T***, Nikopensius T*, Krjutškov K, Tammekivi V, Viltrop T, Saag M, Metspalu A. MTHFR and MSX1 contribute to the risk of non-syndromic cleft lip/palate. *Eur J Oral Sci* 2010; 18: 213–220.

III. Nikopensius T*, **Jagomägi T***, Krjutškov K, Tammekivi V, Saag M, Prane I, Piekuse L, Akota I, Barkane B, Krumina A, Ambrozaitytė L, Matulevičienė A, Kučinskienė ZA, Lace B, Kučinskas 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: 748–756

IV. Nikopensius T, Kempa I, Ambrozaitytė L, **Jagomägi T**, Saag M, Matulevičienė A, Utkus A, Krjutškov K, Tammekivi V, Piekuse L, Akota I, Barkane B, Krumina A, Klovins J, Lace B, Kučinskas V, Metspalu A. Variation in FGF1, FOXE1, and TIMP2 genes is associated with non-syndromic cleft lip with or without cleft palate. *Birth Defects Res A Clin Mol Teratol* 2011; 91: 218–225.

* These authors contributed equally to this work.

Author's contribution:

I. The author collected and analyzed all materials. The author wrote the manuscript.

II. The author collected all materials for the study. The author wrote the manuscript.

III. The author collected all materials from the Estonian side. The author was one of the writers of the manuscript.

IV. The author collected all materials from the Estonian side. The author was one of the writers of the manuscript.

LIST OF ABBREVIATIONS

ABCA	ATP-binding cassette, sub-family
ADH	alcohol dehydrogenase
APOC	Apolipoprotein
BCL	B-cell CLL/lymphoma
BCLP	bilateral cleft lip and palate
BMP	bone morphogenetic protein
CCDC	coiled-coil domain containing
CDH	Cadherin
CEU	U.S. residents (Utah) with northern and western European ancestry
CHD7	chromodomain helicase DNA-binding protein 7
CL	cleft lip only (excludes [1] cleft lip and alveolus, [2] cleft lip and palate, and [3] cleft palate)
CLF	cytokine receptor-like
CLP	cleft lip and palate (excludes [1] cleft lip and [2] cleft palate)
CL/P	cleft lip and/or cleft palate = cleft lip + cleft lip and palate + cleft palate (no exclusions)
CLPTM	cleft lip and palate transmembrane
COL	Collagen
CP	cleft palate (excludes [1] cleft lip and [2] cleft lip and palate)
CPO	cleft palate only
CRISPLD	cysteine-rich secretory protein LCCL domain containing
CYP	cytochrome P450
DHCR	dehydrocholesterol reductase
DNA	deoxyribonucleic acid
ECE	endothelin converting enzyme
EDN(R)	endothelin (receptor)
EDTA	ethylene diamine tetraacetic acid
EUROCAT	European network of population-based registries for the epidemiologic surveillance of congenital anomalies
FGF	fibroblast growth factor
FGF(R)	fibroblast growth factor (receptor)
FOXC	forkhead box C
FOXE	forkhead box protein E
GLI	zinc finger protein
GSTM	glutathione S-transferase-Mu
GSTT	glutathione S-transferase theta
GWA	genome-wide association
HapMap	haplotype map
HEMGN	Hemogen
ICBDSR	International Clearinghouse for Birth Defects Surveillance and Research
IPDTOC	International Perinatal Database of Typical Orofacial Clefts

IRF	interferon regulatory factor
JAG	protein jagged
Kb	kilobase, 1.000 base pairs
L	Linkage
LD	linkage disequilibrium
LHX	LIM homeobox
M	mutation detection
MAF	minor allele frequency
MAFB	v-maf musculoaponeurotic fibrosarcoma oncogene homolog B
MMP	matrix metalloproteinase
MSX	muscle segment homeobox
MTHFR	methylenetetrahydrofolate reductase
MYH	myosin, heavy chain 9, non-muscle
NAT	N-acetyltransferase
NSOC	nonsyndromic oral cleft
OC	oral cleft
OFC	orofacial cleft
OMIM	Online Mendelian Inheritance in Man
OR	odds ratio
P63	tumour protein p63
PAX	paired box
p.c.wk	post-coital week
PDGFC	platelet derived growth factor C
PHF	PHD finger protein
PLINK	whole genome association analysis toolset
PPS	popliteal pterygium syndrome
PQBP	polyglutamine binding protein
PTCH	protein patched homolog
PVR (L)	poliovirus receptor (like)
RARA	retinoic acid receptor, alpha
RYK	receptor-like tyrosine kinase
SATB	SATB homeobox
SHH	sonic hedgehog homolog
SKI	v-ski sarcoma viral oncogene homolog
SMIR	SMAD-IRF-binding domain
SNP	single nucleotide polymorphism
tag SNP	haplotype-tagging SNP
SOX	sex determining region Y
SPRY	sprouty homolog
SPSS	Statistical Package for the Social Sciences
SUMO	small ubiquitin-related modifier
TBX	T-box transcription factor
TCOF	Treacher Collins-Franceschetti syndrome
TFAP	transcription factor activating enhancer binding protein
TGF	transforming growth factor

TIMP	tissue inhibitor of matrix metalloproteinases
UCSC	University of California, Santa Cruz
VAX	ventral anterior homeobox
VWS	van der Woude syndrome
WNT	wingless-type MMTV integration site family

INTRODUCTION

Cleft lip (CL), cleft lip with or without cleft palate (CL/P) and isolated cleft palate (CP), collectively termed oral clefts (OC), are the second most common birth defects among newborn. These defects arise in about 1 in 700 liveborn babies, with ethnic and geographic variation. Approximately 75% of CL/P and 50% of CP cases are isolated defects and no other deformities are found in those children. Those OCs are therefore called nonsyndromic (Stainer and Moore, 2004).

Although OC is usually not a life-threatening condition, many functions such as feeding, digestion, speech, middle-ear ventilation, and hearing, respiration, facial and dental development can be disturbed because of the structures involved. These problems can also cause emotional, psychosocial and educational difficulties. Affected children need multidisciplinary care from birth until adulthood (Mossey et al., 2009). Orofacial clefts pose a burden to the individual, the family, and society, with substantial expenditure, and rehabilitation is possible with good quality care. Care for children born with these defects generally includes many disciplines – nursing, facial plastic surgery, maxillofacial surgery, otolaryngology, speech therapy, audiology, counselling, psychology, genetics, orthodontics, and dentistry. Fortunately, early and good quality rehabilitation of children with OC usually gives satisfactory outcomes.

Identification of etiological factors for OC is the first step towards primary prevention. Genetic factors contributing to CL/P formation have been identified for some syndromic cases, but knowledge about genetic factors that contribute to nonsyndromic CL/P is still unclear. The high rates of familial occurrences, risk of recurrence, and elevated concordance rates in monozygotic twins provide evidence for a strong genetic component in nonsyndromic CL/P. However, concordance in monozygotic twins ranges between 40% and 60%, which means that the exact inheritance pattern of OC is more complex. It has been suggested that the development of nonsyndromic OC occurs as a result of the interaction between different genetic and environmental factors (Mitchell and Risch, 1992; Carinci et al., 2000; Carinci et al., 2003; Carinci et al., 2007). The identification of the genes responsible for diseases has been a major focus of human genetics over the past 40 years. The introduction of modern molecular methods, experimental animal knockout models and advances in the technique of gene mapping have provided new candidate genes for orofacial clefting, both for syndromic and nonsyndromic cases. However, the results of earlier candidate-gene-based association studies, performed in different populations, have been conflicting, with only a few candidate loci being implicated in OC phenotypes. This inconsistency indicates the challenges in searching associations with a relatively rare phenotype such as nonsyndromic clefting.

The primary purpose of the present study was to give an overview of OC in Estonia. National statistics regarding orofacial clefts in Estonia was nonexistent at the start of this study. The only prevalence findings known to us were those of Lõvi-Kalnin (1996), conducted during 1970–1980. On the basis

of the data from the study, the current rate of occurrence of clefts in Estonia would be 1 case per 777 live births. The only way to estimate the number of children affected by clefts is to use the pre-existing information from previous visits to maxillofacial surgeons. Genotype-phenotype correlation research in this area could yield important information on risk factors. Therefore, our second main purpose was to investigate the possible contributions of recognized candidate genes, on the basis of multiple reports on the association between markers and haplotypes in various genes and orofacial clefts from a previously uncharacterized ethnic background of three genetically close populations from Estonia, Latvia and Lithuania.

REVIEW OF LITERATURE

1. Embryonic development

The development of the head (Figure 1) involves the interaction of several cell populations and the coordination of cell signalling pathways which, when disrupted, can cause defects such as facial clefts. Development of the lip and palate entails a complex series of events (Table 1). Disturbances at any stage during palate development (e.g., defective palatal shelf growth, failed or delayed elevation, and blocked fusion) can result in CP/L or CP only (Sperber, 2010).

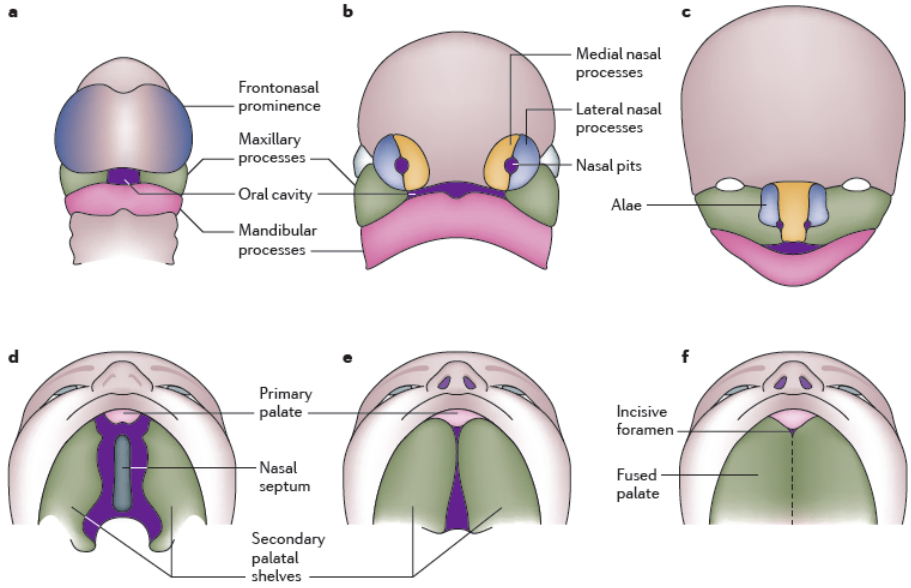


Figure 1. Development of the lip and palate

a. The developing frontonasal prominence, paired maxillary processes and paired mandibular processes surround the primitive oral cavity by the 4th week of embryonic development.
b. By the 5th week, the nasal pits have formed, which leads to the formation of the paired medial and lateral nasal processes.
c. The medial nasal processes have merged with the maxillary processes to form the upper lip and primary palate by the end of the 6th week. The lateral nasal processes form the nasal alae. Similarly, the mandibular processes fuse to form the lower jaw.
d. During the 6th week of embryogenesis, the secondary palate develops as bilateral outgrowths from the maxillary processes, which grow vertically down the side of the tongue.
e. Subsequently, the palatal shelves elevate to a horizontal position above the tongue, contact one another and commence fusion.
f. Fusion of the palatal shelves ultimately divides the oronasal space into separate oral and nasal cavities (Dixon et al., 2011).

Table 1. Chronology of key embryonic events (from Sperber, 2010, with permission)

Carnegie Stage	Postconception Age	Craniofacial Features
6	14 days	Primitive streak appears; oropharyngeal membrane forms
8	17 days	Neural plate forms
9	20 days	Cranial neural folds elevate; otic placode appears
10	21 days	Neural crest migration commences; fusion of neural folds; otic pits forms
11	24 days	Frontonasal prominence swells; first arch forms; wide stomodeum; optic vesicles form; anterior neuropore closes; olfactory placode appears
12	26 days	Second arch forms; maxillary prominence appear; lens placodes commence; posterior neuropore closes; adenohypophysial pouch appears
13	28 days	Third arch forms; dental lamina appears; fourth arch forms; oropharyngeal membrane ruptures
14	32 days	Otic and lens vesicles present; lateral nasal prominence appear
15	33 days	Medial nasal prominence appear; nasal pits form-widely separated, face laterally
16	37 days	Nasal pits face ventrally; upper lip forms on lateral aspect of stomodeum; lower lip fuses in midline; retinal pigment forms; nasolacrimal groove appears, demarcating nose; neurohypophysial evagination
17	41 days	Contact between medial nasal and maxillary prominence, separating nasal pit from stomodeum; upper lip continuity first established; vomeronasal organ appears
18	44 days	Primary palate anlagen project posteriorly into stomodeum; distinct tip of nose develops; eyelid fold form; retinal pigment; nasal pits move medially; nasal alae and septum present; mylohyoid, geniohyoid and genioglossus muscle form
19	47–48 days	Nasal fin disintegrates; (failure of disintegration predisposes to cleft lip); the rima oris of the mouth diminishes in width; mandibular ossification commences
20	50–51 days	The lidless eyes migrate medially; nasal pits approach each other; ear hillocks fuse
22	54 days	The eyelids thicken and encroach upon the eyes; the auricle forms and projects; the nostrils are in definite position
23	56–57 days	Eyes are still wide apart but eyelid closure commences; nose tip elevates; face assumes a human fetal appearance; head elevates off the thorax; mouth opens; palatal shelves elevate; maxillary ossification commences
Fetus	60 days	Palatal shelves fuse; deciduous tooth buds form; embryo now termed a fetus

1.1. Lip development

During the third week of gestation, neural crest cells proliferate and migrate into the frontonasal and visceral arch region to form the five facial primordia. By the post-coital fourth week (p.c.4wk), five primordia (consisting of the frontonasal prominence, the paired maxillary prominences, and the paired mandibular prominences) surround the primitive oral cavity. The frontonasal prominence forms the forehead and the nose. The maxillary prominences are bilateral and form the lateral stomodeum (primitive mouth). The mandibular prominences are also bilateral and are responsible for the caudal growth of the stomodeum. The neural crest cells within these prominences differentiate into skeletal and connective tissue of the face, bone, cartilage, fibrous connective tissue, and all dental tissues except enamel (Sperber, 2010). During the fourth week (p.c.4wk), the medial ends of the mandibular prominences merge to form the mandible, lower lip, and lower cheek region. Toward the end of the fourth week, nasal placodes form on the lower aspect of the frontonasal prominence. The nasal or olfactory pits form and extend into the primitive mouth, and these become the nostrils. Rapid growth continues in weeks five to six (p.c.5–6wk). In weeks six to seven (p.c.6–7wk), rapid proliferation of the maxillary prominences results in the medial nasal prominences merging with each other and the lateral nasal prominences to form the lateral nose and cheek regions. The upper lip is formed during this period by the lateral movement of the maxillary prominences and medially by the fused medial nasal prominences (Moore and Persaud, 2008).

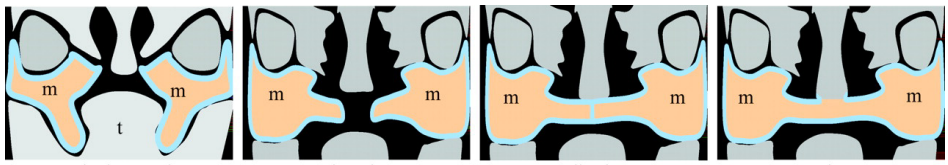
1.2. Palate development

Palate development begins during the post-coital fifth week (p.c.5wk), after fusion of the upper lip, and is complete at the end of the twelfth week. Palatal development follows up the initial development of the oral region with further proliferation and migration of the maxillary prominences. Development of the palate is divided into two regions: the primary and secondary palate. Development of the primary palate starts with further development of the intermaxillary segment of the maxilla. This tissue is derived from the maxillary prominences merging with the medial nasal prominences. The intermaxillary segments of the maxilla form the labial components that form the philtrum of the upper lip and the bony palatal component. The primary palate extends posteriorly to the incisive foramen, located immediately behind the alveolar ridge (Moore and Persaud, 2008).

The secondary palate originates as an outgrowth of the maxillary prominences (p.c.6wk). During the seventh week of development, the palatal shelves rise to a horizontal position above the tongue, and come into contact and fuse to form a midline epithelial seam, which subsequently degenerates to allow mesenchymal continuity across the palate. The palatal mesenchyme then differentiates into bony and muscular elements that correlate with the position of the

hard and soft palate respectively. With continued growth, the shelves appose at the midline (p.c.10wk) and eventually fuse (p.c.13wk) (Murray and Schutte, 2004).

Numerous genes are expressed during palatal development (Figure 2).



Vertical Growth | Elevation | Adhesion | Fusion

Mesenchyme	Epithelium	Mesenchyme	Epithelium	Mesenchyme	Epithelium	Mesenchyme
EphB2/B3[10]	pERK[5]	EphB2/B3[10]	pERK[5]	Bmp2, 3, 4[20]	BMP3[9]	Bmp2,3,4[20]
Fgf10[22]	Fgfr2b[22]	Fgf10[22]	Fgfr1[3]	EphB2/B3[10]	CS-PG[12]	Lhx8[28]
Fgf7[22]	pMEK[5]	Fgfr1, 2b[22]	Fgfr2b[22]	Fgf10[21]	EphB2/B3[10]	MMP2[4]
Fz4[14]	PI3K[5]	$GABA_A\beta3$[13]	$GABA_A\beta3$[13]	Lhx8[28]	Fgf2[3]	Osr2[16]
Gli1, 2, 3[24]	pPTEN[5]	Lhx8[28]	pMEK[5]	MMP2, 13[1,4]	Fgfr2[3]	Snail[19]
Id2[23]	Shh[24]	Msx1[18]	PI3K[5]	Msx1[18]	$GABA_A\beta3$[13]	
Lhx8[28]	TBX22[2]	Osr2[16]	pPTEN[5]	PI3K[5]	IRF6[15]	
Msx1[18]	TBX1[29]	PI3K[5]	Ptc2[24]	Shox2[23, 26]	MMP-13[1]	
Osr2[16]		Shox2[18, 26]	Shh[24]	Snail[23, 19]	MT1-MMP[1]	
Pax9[16]		Snail[23,19]	Shox2[26]	Twist[23, 27]	pMEK[5]	
Pdgfrα[25]		Twist[23,27]	TBX1[29]		PI3K[5]	
PI3K[5]		TGFβ1,2[3]	TGFβ1,2[21]		pPTEN[5]	
Ror2[14]			Wnt11[17]		Ptc2[24]	
SATB2[9]					Shh[24]	
Shox2[18,26]					Shox2[26]	
Snail[23,19]					Smad2[8]	
TBX22[2]					Snail[19]	
Twist[23]					STAT[3]	
Wnt5a[14]					TBX1[29]	
					TIMP-2[1]	
					Tgfβ3[3]	
					TgfbrII[6], III[7]	
					Twist[27]	
					Wnt11[17]	
					Zeb1(Zfhx1a)[9]	

Figure 2. Gene expression during different stages of palatal development
The schematic drawing shows coronal view of normal palate shelf and key stages of palatal development. Main genes that express and function at each palatal developmental stages are summarized. Genes are separated according to the location of its expression pattern (mesenchyme vs. epithelium) (Wenli Yu et al., 2009).

1: Blavier L. et al, 2001, Mol Biol Cell; 2: Braybrook C. et al, 2002, Hum Mol Genet; 3: Britto JA. et al, 2001, Cleft Palate Craniofac J; 4: Brown NL. et al, 2002, J Dent Res; 5: Cho KW. et al, 2008, Gene Expr Pattern; 6: Cui XM et al, 1998, Int J Dev Biol; 7: Cui XM. et al, 2000, Int J Dev Biol; 8: Cui SM. et al, 2003, Dev Biol; 9: Dean D. et al, 2008, Development; 10: McLean W. et al, 2008, Mech Dev; 11: FitzPatrick DR. et al, 2003, Hum Mol Genet; 12: Gato A. et al. 2002. Dev Biol; 13: Hagiwara N. et al, 2003, Dev Biol; 14: He F. et al, 2008, Development; 15: Kondo S. et al, 2002, Nat Genet; 16: Lan Y, et al, 2004, Development; 17: Lee J-M. et al, 2008, Dev Biol; 18: Li Q. et al, 2007, Int J Dev Biol; 19: Martínez-Álvarez C. et al, 2004, Dev Biol; 20: Nie XG. et al, 2006, Int J Dev Biol; 21: Pelton RW. et al, 1990, Dev Biol; 22: Rice R. et al, 2004, J Clin Invest; 23: Rice R. et al, 2005, Dev Dyn; 24: Rice R. et al, 2006, Gene Expr Patterns; 25: Xu X. et al, 2005, Dev Dyn; 26:Yu L., et al, 2005 Development; 27: Yu W. et al, 2008, Dev Dyn; 28: Zhao YG. et al, 1999, PNAS; 29: Zoupa M., 2006, Int J Dev Biol.

Mice and chicks have played a central part in dissection of the molecular pathways underlying development of the lip and palate. In both species, development of the lip and primary palate closely parallels that seen in human beings (Jiang, 2006).

2. Classification of clefts

Various classification schemes for OC have been devised in the last 70 years, but few have received widespread clinical acceptance. OC can be classified on the basis of etiology and/or pathogenesis. There have been multiple classifications of OC based on anatomic and embryological considerations.

2.1. Fogh-Andersen classification (1942)

Fogh-Andersen divided OC as follows:
CL extending to the incisive foramen, including clefts of the alveolus
CL and CP (CLP), including unilateral and bilateral CLP
CP identified as being median and not extending beyond the incisive foramen.

2.2. Davis and Ritchie classification (Davis and Ritchie, 1922)

The Davis and Ritchie classification divides CL/P into two groups, which are subdivided by the extent of the cleft (eg, 1/3, 1/2), as follows:

Group I – Clefts anterior to the alveolus (unilateral, median, or bilateral cleft lip).

Group II – Postalveolar clefts (cleft palate alone, soft palate alone, soft palate and hard palate, or submucous cleft).

2.3. Veau classification (Figure 3)

Group I – Defects of the soft palate only.

Group II – Defects involving the hard palate and soft palate.

Group III – Defects involving the soft palate to the alveolus, usually involving the lip.

Group IV – Complete bilateral clefts.

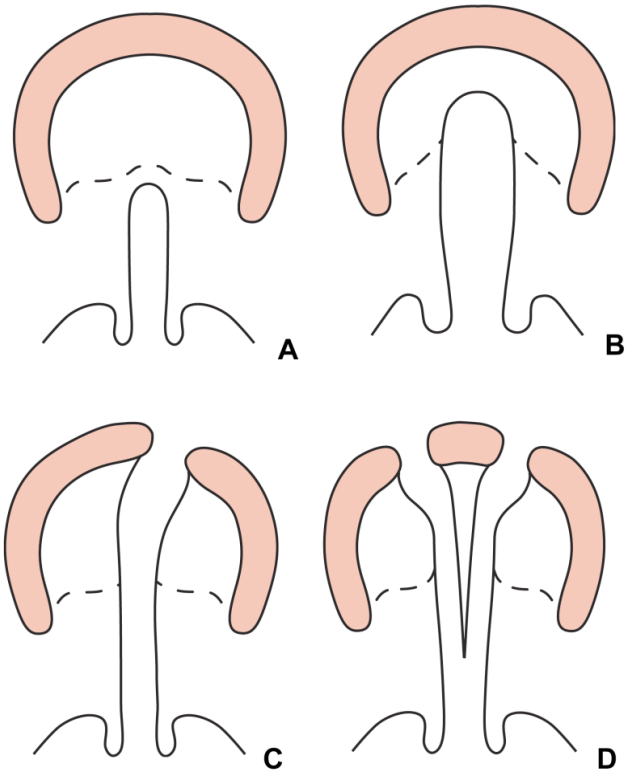


Figure 3. Veau classification
A: Group I. Defects of the soft palate only. B: Group II. Defects involving the hard palate and soft palate. C: Group III. Defects involving the soft palate to the alveolus, usually involving the lip. D: Group IV. Complete bilateral clefts (Tewfik et al., 2011).

2.4. Kernahan and Stark classification (Figure 4)

The Kernahan and Stark classification highlights the anatomic and embryonic importance of the incisive foramen. This system provides a graphic classification scheme using a Y-configuration, which can be divided into nine areas, as follows:
Areas 1 and 4 – Lip
Areas 2 and 5 – Alveolus
Areas 3 and 6 – Palate between the alveolus and the incisive foramen
Areas 7 and 8 – Hard palate
Area 9 – Soft palate

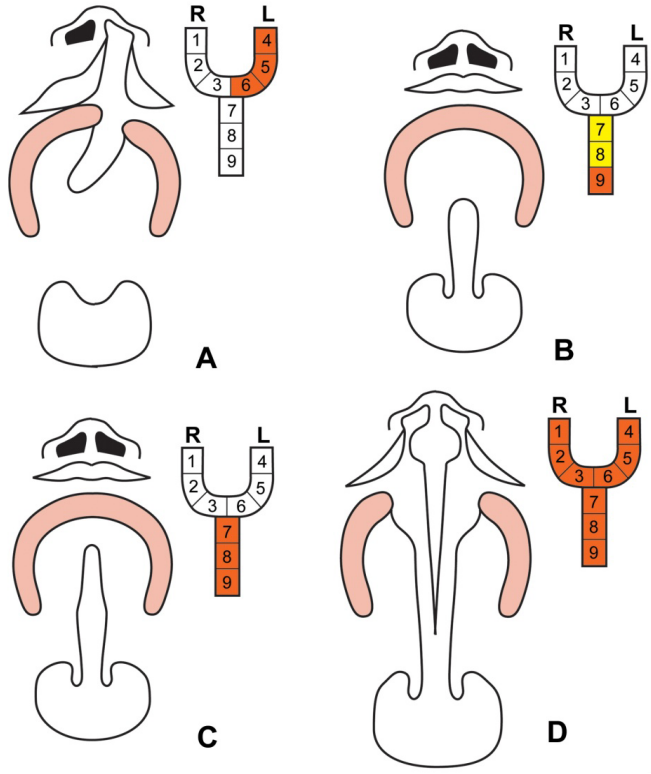


Figure 4. Kernahan and Stark classification
Areas 1 and 4 – Lip; Areas 2 and 5 – Alveolus; Areas 3 and 6 – Palate between the alveolus and the incisive foramen; Areas 7 and 8 – Hard palate; Area 9 – Soft palate. R = right; L = left (Kernahan, 1971).

2.5. International Confederation for Plastic and Reconstructive Surgery classification:

The International Confederation for Plastic and Reconstructive Surgery (1967) established a classification of OC based on the embryology of the developing structures (Millard, 1976).

- Clefts of the primary palate
  Lip
  Alveolus
- Clefts of the primary and secondary palate
  Lip
  Alveolus
  Hard palate (secondary palate)
- Clefts of the secondary palate
  Hard palate
  Soft palate

Classifications of OC based on etiology or pathogenesis provide insufficient information regarding the severity of the cleft and the types of direct medical and surgical management required. It is necessary to combine the classifications in order to optimally diagnose and treat cleft patients (Figure 5).

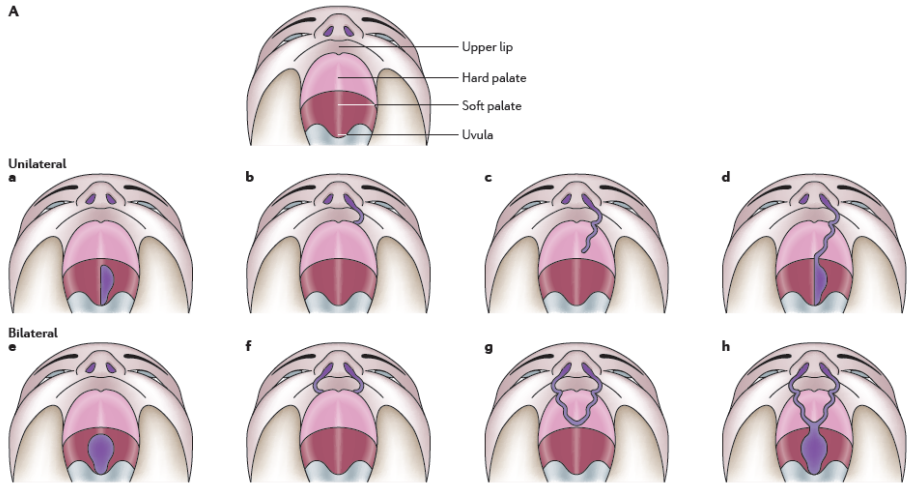


Figure 5. Types of clefts
a. unilateral cleft of the soft palate; b. unilateral cleft lip; c, d unilateral cleft lip and palate; e. bilateral cleft of the soft palate; f. bilateral cleft lip; g,h. bilateral cleft lip and palate. Clefts are indicated in purple (Dixon et al., 2011).

3. Prevalence

3.1. CL/P prevalence

According to the International Perinatal Database of Typical Orofacial Clefts (IPDTOC, 2011) the overall prevalence of CL/P is 9.92 per 10,000 worldwide. The prevalence of CL is 3.28 per 10,000 and that of cleft lip and palate (CLP) 6.64 per 10,000. According to the IPDTOC study (2011), in the evaluation of geographical areas, the registries in Japan, Mexico, South America, Western Europe and Canada have a higher prevalence of CL/P than the overall estimate, while the registries in Eastern Europe, South-Mediterranean Europe and South Africa reported a lower prevalence.

Asian and Native American populations have the highest reported birth prevalence rates, which are often as high as 1 in 500. European-derived populations have intermediate prevalence rates at approximately 1 in 1,000, and African-derived populations have the lowest prevalence rates at approximately 1 in 2,500 (Dixon et al., 2011).

3.2. CP prevalence

CP is a common congenital anomaly affecting about 1 in 2,000 livebirths worldwide. Most CP cases occur as an isolated congenital malformation, but they are often part of chromosomal aberrations and monogenic syndromes or are associated with other congenital malformations (Shaw et al., 1995; Schutte and Murray, 1999; Beaty et al., 2002).

The prevalence of CP varies significantly in Europe, not only between registries but also within countries, with a European mean value of 6.2/10,000 (EUROCAT, 1995, 1997, 2002). The highest prevalence (14.2/10,000) of isolated CP was confirmed in Finland (Finland National Institute for Health and Welfare, 2011).

4. Cleft proportion

The proportion of different types of cleft is providing clues about the underlining etiology. In most of the studies from Europe and the U.S. about nonsyndromic OC, unilateral CLP is the most frequent, accounting for about 30–35% of cases. Isolated CL and CP each account for between 20–25% and (BCLP) is about 10% (Wyszynski, 2002).

The left unilateral cleft is a most common finding and seems to be a common feature in all ethnic groups (Tolarova, 1987). The left side is affected twice as often as the right side (Dixon et al., 2011).

Fogh-Andersen (1942) reported a CL:CLP:CP ratio of 1:2:1, which is often described as the normal ratio for different cleft types in Caucasians. Studies from Japan and Africa (Natsume and Kawai, 1986; Ogle, 1993) reveal a much lower prevalence of CP.

5. Gender ratio

The gender ratio among individuals with CL/P is distorted in the general population, with males being affected two times more frequently than females. The opposite situation, a significantly higher incidence of females compared to males, is found for CP (EUROCAT, 200; Dixon et al., 2011). No generally accepted explanation for the sex differences is reported. The discovery of the X-linked CP and ankyloglossia gene *TBX22* (Braybrook, 2001; Marçano et al., 2004) may suggest a candidate gene that might be relevant for palate morphogenesis and possibly play a role in the imbalanced sex prevalence of CP cases (Marçano et al., 2004).

6. Surveillance system

National statistics regarding OC in Estonia are non-existent. Currently, the only way to estimate the number of children affected by clefts is to use the pre-existing information from previous visits to maxillofacial surgeons. The data

retrieved in this way can be significantly distorted, as patients have an opportunity to carry out surgical procedures outside Estonia.

We used the Health Insurance Fund's diagnosis code database to identify primary cases of clefts. Unfortunately, doctors use codes in different ways and a first-time patient's code is reused on recurring patients over many years.

Due to a lack of official data on clefts in Estonia, national data is also absent in different organizations' overviews.

7. Specific exposures

Population-based studies have shown that non-genetic factors play an important role in clefting.

7.1. Cigarette smoking

The proportion of clefts attributable to maternal smoking in populations with a high prevalence of smoking in women of reproductive age was estimated at 22% (Little et al., 2004a). In many countries, tobacco use is rapidly increasing in women of reproductive age because they are actively targeted by tobacco marketing campaigns (Windsor, 2002). Several studies have demonstrated conflicting results. Maternal smoking has been linked to CL/P in offspring (Little et al., 2004b; van Rooij et al., 2001; Wyszynski, 2002a,b). A different study has linked smoking during pregnancy to dose-related OC in newborns (Wyszynski, 2002a,b). Another study found an association between maternal smoking and CP, but not maternal smoking and CL/P (Meyer et al., 2004). In addition, it is possible that there may be a strong interaction between certain maternal and/or infant gene variants and smoking leading to OC in an infant (Shaw et al.,1996; Fallin et al., 2003; Lammer et al., 2004a). A gene-environment interaction has been found in the occurrence of OC in children with the rare allele C2 of the transforming growth factor alpha (*TGF-α*) gene, who were born to women who smoked during pregnancy (Shaw et al.,1996).

7.2. Medication and drugs

Epidemiological studies have linked certain drugs during pregnancy to a higher risk of having a child with OC. Maternal intake of vasoactive drugs, such as pseudoephedrine, aspirin, ibuprofen, amphetamine, cocaine, or ecstasy**,** have been associated with a higher incidence rate for OC (Beaty et al., 1997; Lammer et al., 2004b). Anticonvulsant medications such as phenobarbital, valproate, trimethadione and dilantin have been documented as increasing the incidence of CL/P (Harden, 2009; Holmes et al., 2004; Källen, 2003; Wyszynski and Beaty, 1996). However, there is some question as to whether this increase is due to the medications or the underlying epilepsy (Wyszynski and Beaty, 1996). The association between antiepileptic drugs and OC may be related to the fact that

many antiepileptic drugs reduce plasma folate levels (Schwaninger et al., 1999). Isotretinoin has been identified as a potential causative factor for OC (Benke, 1984; Lammer et al., 1985). Diazepam (Valium) and Bendectin have not been found to increase the rate of OC (Mitchell et al., 1981; Rosenberg et al.,1983). An association between maternal intake of sulfasalazine, naproxen, and glucocorticoids during the first trimester and clefting in newborn has been suggested (Källen, 2003). Aminopterin (an anticancer drug) has also been linked to the development of OC (Warkany, 1978).

Corticosteroids are first-line drugs used to treat a variety of conditions in women of childbearing age; in animal models the role of corticosteroids in clefting is well documented.

Corticosteroids, either used topically or systemically, have shown a weak association with an increased risk of orofacial clefting (Edwards et al., 2003; Pradat et al., 2003).

7.3. Alcohol

The teratogenicity of alcohol has been demonstrated in animal models (Cudd, 2005). It has been established that alcohol is a teratogen in humans, most clearly in the etiology of fetal alcohol syndrome. Several studies have shown that maternal alcohol intake may increase the risk of OC (Lorente et al., 2000a; Shaw et al., 1999a). However, this association was not repeated in another study (Meyer et al., 2003). Almost all studies have used different definitions for alcohol intake but, despite these differences, these studies consistently demonstrated, with only a few exceptions, an association between high alcohol intake and an increased risk of CL/P (Lorente et al., 2000a; Shaw et al., 1999a). Heavy drinking during pregnancy is uncommon, and the small numbers of exposed women in many studies have made it difficult to assess this association. A possible mechanism for alcohol-induced embryonic malformations is ethanol inhibition of retinoic acid synthesis during embryogenesis (Kot-Leibovich and Fainsod, 2009). When consumed at high levels, ethanol competitively inhibits the production of retinoic acid which is necessary for normal cranial neural crest development.

Studies have found that the infant *ADH1C* (alcohol dehydrogenase) genotype is associated with the risk of CL/P (Jugessur et al., 2009; Boyles el al., 2010). Alcohol-metabolizing genes are expressed in placental tissue during the first trimester of pregnancy (Edenberg et al., 2006), when the critical stages of facial development occur.

7.4. Diet and vitamins

It has been suggested that nutrition plays a role in the manifestation of OC. Maternal periconceptional use of folic acid has been found to reduce the risk of neural tube defects. As a result, the question has been raised about whether there is a similar protective effect for other birth defects, including OC.

Maternal multivitamin use has been found to result in a significant reduction in CP risk and a nonsignificant reduction in CL risk (Werler et al.,1999). Several studies have shown decreased rates of CL/P cleft lip and palate with folic acid use (Malek et al., 2003; Shaw et al., 1995; Shaw et al., 2002, Tolarova and Harris, 1995), while other studies have failed to find such an effect (Hayes et al., 1996). Some ambiguity in the studies may be explained by a study that found OC risk can be reduced only by consuming high doses of folic acid at the time of lip and palate formation (Czeizel et al., 1999). A reduction in the risk of OC has been reported with the use of zinc and B vitamins (Munger et al., 2004, Krapels et al., 2004), as well as vitamin A (Mitchell et al., 2003). In addition, offspring of mothers with the *MTHFR* 677TT or *MTHFR* 1298CC genotype and low periconceptional folate intake were found to have an increased risk of CL/P (Jugessur et al., 2003b; van Rooij et al., 2003).

7.5. Solvents and pesticides

Maternal occupational exposure to glycol ethers, a chemical found in various domestic and industrial products, has been found to increase the rate of CL (Cordier et al., 1997). Exposure to organic solvents (xylene, toluene, acetone) has also been reported to increase the rate of this defect (Wyszynski and Beaty,1996). Maternal occupations involving hazardous chemicals such as hairdressing**,** agriculture, and leather or shoe manufacturing, as well as exposure to pesticides, lead, and aliphatic acids, have been reported to increase rates of OC (Garcia and Fletcher, 1998; Lorente et al., 2000; Wyszynski and Beaty, 1996); however, other studies failed to find a link between pesticides and OC risk (Shaw et al., 1999a; Wyszynski and Beaty, 1996). One study (Irgens et al., 1998) failed to find any link between parental occupational exposure to lead and OC risk. However, the number of cases in the study was small, and the lead exposure was measured by census records. Maternal exposure to general laboratory chemicals was not seen as significant, although exposure to organic solvents, specifically benzene, was found to be a contributing factor in the increase of neural crest malformations in offspring, including orofacial clefting (Wennborg et al., 2005).

Living in proximity to hazardous waste facilities does not appear to increase the risk for CL/P (Croen and Shaw, 1997), nor does parental occupational exposure to 50 Hz magnetic fields (Blaasaas et al., 2002). Studies have been unable to find conclusive evidence of an effect from exposure to water chlorination and chlorination byproducts (Hwang and Jaakkola, 2003).

One study (Shaw et al., 1999b) found that periconceptional use of electric bed-heating devices (electric blankets, bed warmers, and heated waterbeds) did not appear to affect the risk of OC. Maternal fever was associated with increased risk, but the intake of multivitamin supplements appeared to lower this risk (Botto et al., 2002).

8. Parental age

Several studies have reported an increased risk of OC with increased maternal age (Shaw et al., 1991). However, larger studies have failed to identify advanced maternal age as a risk factor for OC (Abramowicz et al., 2003, Vallino-Napoli et al., 2004, González et al., 2008). Conversely, another study found a greater risk for CL among younger mothers (Reefhuis and Honein, 2004).

9. Genetic approaches

To date, genetic approaches to nonsyndromic CLP have included: linkage analysis; association studies; identification of chromosomal anomalies or microdeletions in cases; and direct sequencing of DNA samples from affected individuals (Dixon et al., 2011).

These methods can be applied to candidate genes or genome-wide strategies can be used. Each approach has its own advantages and disadvantages, some of which will depend on the underlying genetic architecture of the disease, as well as the realities of economics and technology.

9.1. Linkage studies

Findings of linkage studies have suggested various loci could have a causal role in CL/P, including regions on chromosomes 1, 2, 4, 6, 14, 17, and 19 (*MTHFR*, *TGF-α*, *D4S175*, *F13A1, TGF-β3*, *D17S250*, and *APOC2*), with putative loci suggested at 2q32–q35 and 9q21–q33 (Marazita et al., 2004a). Inconsistent results could be caused by the small size of the studies or genetic heterogeneity.

9.2. Association studies

Various genetic polymorphisms have been investigated in population-based association studies. Some genes function as growth factors (eg, *TGF-α*, *TGF-β3*), transcription factors (*MSX1, IRF6, TBX22*), or factors that play a part in xenobiotic metabolism (*CYP1A1, GSTM1, NAT2*), nutrient metabolism (*MTHFR, RARA*) or immune response (*PVRL1, IRF6*) (Mossey et al., 2009). The most intensively investigated genes have been the *TGF-α* (Mitchell, 1997; Zeiger et al., 2005; Vieira, 2006) and *MTHFR* (Chevrier et al., 2007; Vieira et al., 2005a) genes.

Inconsistent data have demonstrated the challenges of researching gene-disease associations and related interactions. However, *IRF6* has shown consistent evidence of association with CL/P across populations of different ancestry (Zucchero et al., 2004; Park et al., 2007; Jugessur et al., 2008; Rahimov et al., 2008).

9.3. Identification of chromosomal anomalies or microdeletions

Analysis of chromosomal anomalies in patients has proven to be a productive route for the identification or confirmation of CL/P loci, with recent successes for *FGFR2* (Osoegawa et al., 2008) and *SUMO1* (Alkuraya et al., 2006; Shi et al., 2009; Mostowska et al., 2010).

9.4. Targeted resequencing studies

Targeted resequencing studies of candidate genes have found specific variants that might underlie associations with clefting, with the strongest current evidence for mutations in *MSX1* (Jezewski et al., 2003; Vieira et al., 2005b), *FGFR1* and *FGF8* (Riley et al., 2007a; Riley et al., 2007b), and *BMP4* (Suzuki et al., 2009). Resequencing studies have also identified private missense mutations as rare causes of nonsyndromic orofacial clefts, including *TGF-β3* (Lidral et al.,1998); *MSX1* (Jezewski et al., 2003; Vieira et al., 2005b); *TBX22* (Marçano et al., 2004); *FOXE1*, *GLI2, JAG2, LHX8, MSX2, SKI, SPRY2*, and *TBX10* (Vieira et al., 2005b); *PTCH1* (Mansilla et al., 2006); *PVR* and *PVRL2* (Warrington et al., 2006); *RYK* (Watanabe et al., 2006); FGFs and FGFRs (Riley et al., 2007a; Riley et al., 2007b).

The missense mutations reported in the candidate genes listed above do not clearly segregate in families; suggested reasons for this include variable impact on gene expression levels during embryonic development and incomplete penetrance (Vieira, 2008).

9.5. Genome-wide association (GWA) studies

Findings from recent GWA studies have significantly improved our understanding of genes and pathways that contribute to the etiology of nonsyndromic CL/P. The first GWA scan reported a new major susceptibility locus at chromosome 8q24.21 (Birnbaum et al., 2009), which was independently confirmed in several samples (Grant et al., 2009; Nikopensius et al., 2009; Beaty et al., 2010). No known genes have been reported within the region of association on 8q24.21. According to the University of California, Santa Cruz (UCSC) genome browser, the nearest flanking gene is *CCDC26*, which encodes a retinoic acid-dependent modulator of myeloid differentiation. Because exposure to retinoic acid is known to induce orofacial clefts, *CCDC26* could be considered as a potential candidate gene. Four additional susceptibility loci not previously associated with CL/P that achieved genome-wide significance have been subsequently identified at chromosomes 10q25.3 (*VAX1*), 17q22 (*noggin*), 1p22.1 (*MAFB*) and 20q12 (*ABCA4*) (Nikopensius et al., 2010; Mangold et al., 2010; Beaty et al., 2010).

The lack of consistency in evidence of association for possible candidate loci across studies, where results have mostly been modest, justifies further investigation into the relevance of previous findings, in new samples obtained from a different ethnic background.

10. Genes implicated in lip and palate development

Fogh-Andersen (1942) provided the first population-based evidence that OC has a strong genetic component. Fraser (1970) separated cleft palate only (CPO) and CL/P. There is evidence that families with patients affected by OC have a different genetic background. Conventionally, it has been decided to classify patients with CP only and the remaining patients as CL/P.

The high rates of familial occurrences, recurrence risks, and elevated concordance rates in monozygotic twins provide evidence for a strong genetic component in nonsyndromic CL/P. The disorder has a complex inheritance pattern with no clear mode of inheritance and reduced penetrance, with a positive family history for clefting in approximately one third of patients. A sibling risk ratio of approximately 40 has been reported, and there is a 2–5% increased risk for offspring of affected individuals. Concordance in monozygotic twins ranges between 40% and 60%, but it is only 5% in dizygotic twins (Mitchell and Risch, 1992; Carinci et al., 2000; Carinci et al., 2003; Carinci et al., 2007).

The lack of total concordance in monozygotic twins suggests that genetic factors alone do not fully account for the pathogenesis of the phenotype; this discordance may be a result of either some degree of nonpenetrance, perhaps as a consequence of random developmental events, or environmental influences in utero. However, the highly increased monozygotic twin concordance does strongly support a major genetic component to orofacial clefting (Stanier and Moor, 2004; Mitchell and Risch, 1992).

The advent of gene targeting technology and basic conventional techniques using animal models has led to the identification of genes associated with known and unknown etiologic factors. Animal models, with clefts arising spontaneously or as a result of mutagenesis experiments, provide another exciting avenue for gene mapping. The mouse is an excellent model for studying human clefting because the development of craniofacial structures in these two species is remarkably similar. Whereas CP is a common phenotype in the mouse, CL is rare (Juriloff, 2002). Conservation of genes and linkage relationships between mice and humans is well documented, and the chromosomal location of a gene in humans can often be predicted from its genetic map position in mice. Development of the orofacial complex is very similar between mouse and human embryos, and much of the understanding of developmental mechanisms in humans has been inferred from mice (Diewert and Wang, 1992). It has become evident that CL/P is heterogeneous, and different chromosome regions such as 1q, 2p, 4q, 6p, 14q and 19q have been claimed to contain a clefting locus (Marazita et al., 2004).

10.1. Syndromic forms

There are over 720 known syndromes featuring OC as cardinal symptoms, with etiologies including single-gene defects, teratogens, chromosomal abnormalities and those with uncertain etiology. Most orofacial clefts are believed to be

nonsyndromic, with the rare syndromic cases resulting from factors such as chromosomal abnormalities, characterized Mendelian single-gene syndromes and teratogenic effects (Stanier and Moore, 2004). In approximately 35–50% of CP and 7–15% of CL/P, other physical abnormalities are seen in the affected individual (Gorlin and Cohen, 2001).

Table 2. Syndromic genes associated with cleft lip and palate

Syndrome	Cleft type	Genes	Reference
Apert syndrome	CP	*FGFR2*	Gritli-Linde, 2008; Kreiborg and Cohen, 1992; Martelli et al., 2008; Park et al.,1995; Wilkie et al.,1995; Moloney et al.,1996.
Bamforth-Lazarus syndrome	CP	*FOXE1*	Gritli-Linde, 2008; Castanet et al., 2002; Clifton-Bligh et al., 1998.
Branchio-oculo facial syndrome	CL	*TFAP2A*	Gritli-Linde, 2008; Milunsky et al., 2008.
Ectrodactyly-ectodermal dysplasia-cleft syndrome	CL/P; CP	P63	Gritli-Linde, 2008; Celli et al., 1999; McGrath et al., 2001.
Fetal alcohol syndrome	CL/P	*ADH1B*	Abel, 2006; Green et al., 2007; Seki et al., 2005; Wattendorf and Muenke, 2005.
Hereditary lymphoedema-distichiasis syndrome	CP	*FOXC*	Fang et al.,2000.
Kallmann syndrome	CP	*FGFR1*	Gritli-Linde, 2008; Dode et al., 2007; Dode et al., 2003.
Margarita Island ectodermal dysplasia	CL/P	*PVRL1*	Gritli-Linde, 2008; Suzuki et al., 2000.
Pierre-Robin sequence	CP	*SOX9*	Dixon et al., 2011.
Smith-Lemli-Opitz syndrome	CP	*DHCR*	Gritli-Linde, 2008; Muenke, 2002; Wassif et al., 1998.
Stickler syndrome	CP	*Col11A1, Col11A2, Col2A1*	Dixon et al., 2011; Wilkin et al.,1998.
Treacher Collins syndrome	CP	*TCOF1*	Dixon et al., 2006; Valdez et al., 2004.
van der Woude syndrome	CP; CL/P	*IRF 6*	Gritli-Linde, 2008; Kondo et al., 2002.
X-linked mental retardation with CL/P	CL/P; CP	*PHF8 PQBP1*	Dixon et al., 2011; Gritli-Linde, 2008.

Syndrome	Cleft type	*Genes*	Reference
Crouzon syndrome	CP	*FGFR2*	Dixon et al., 2011; Wilkie et al., 1995.
Unnamed syndrome: Holoprosencephaly 7, a spectrum of forebrain and midline anomalies	CL	*PTCH*	Gritli-Linde, 2008; Muenke, 2002; Ribeiro et al., 2006.
Unnamed syndrome: CP, craniofacial anomalies, osteoporosis, and cognitive defects	CP	*SATB2*	Gritli-Linde, 2008; Leoyklang et al., 2007.
Unnamed syndrome: Holoprosencephaly, a spectrum of anomalies ranging from severe (cyclopia) to subtle midline asymmetries	CL/P	*SHH*	Gritli-Linde, 2008; Muenke, 2002.
Unnamed syndrome: Anomalies with most features of DiGeorge/ velocardiofacial syndromes: thymus and parathyroid gland hypoplasia, vertebra, facial and cardiac outflow anomalies.	CP	*TBX1*	Moreno et al., 2009; Gritli-Linde, 2008; Yagi et al, 2003; Cuneo, 2001.
Unnamed syndrome: X-linked CP and ankyloglossia	CP	*TBX22*	Gritli-Linde, 2008; Dixon et al., 2011.
Loeys-Dietz syndrome	CP	*TGF- β*	Gritli-Linde, 2008; Loeys et al., 2005.

10.2. Nonsyndromic genes

Approximately 75% of CL/P and 50% of CP cases are isolated, nonsyndromic OCs (Tolarova and Cervenka, 1998; Stoll et al., 2000).

Most studies of nonsyndromic clefts to date have focused on CL/P rather than isolated CP. This has been biased perhaps by the larger numbers of cases, easier ascertainment and less confusion from confounding syndromes.

Mutation screens of more than 20 CL/P candidate genes find that 2–6% of the total number of individuals with nonsyndromic CL/P have mutations in genes such as *MSX1, FOXE1, GLI2, JAG2, LHX8, SATB2, RYK1* and others (Jezewski et al., 2003; Vieira et al., 2005b; Watanabe et al., 2006). The large majority of individuals with CL/P (94–98%) do not have mutations in any of a wide range of plausible candidate genes.

The role of genetic factors in determining CP is documented by recurrence risk (Fraser, 1970) and monozygotic twin concordance (Nordstrom et al., 1996), but thus far there is no evidence of any single gene acting as a major factor in

the etiology of malformation. In isolated CP, a major genetic component with a relatively small number of interacting causative loci has been suggested and the final phenotype is the result of gene products that interact in many ways with one another and the environment.

Table 3. Candidate genes for oral clefts (Dixon et al., 2011)

Gene	Location	Evidence	References
Confirmed			
IRF6	1q32	GWA, LD, L, M	Zucchero et al., 2004; Blanton et al., 2005; Ghassibe et al., 2005; Scapoli et al., 2005; Srichomthong et al., 2005; Rahimov et al., 2008; Marazita et al., 2009; Birnbaum et al., 2009.
	8q24 locus	GWA, LD	Birnbaum et al., 2009; Grant et al., 2009; Beaty et al., 2010.
VAX1	10q25	GWA, LD	Beaty et al., 2010; Mangold et al., 2010.
Likely			
ABCA4 (locus only)		GWA	Beaty et al., 2010.
MSX1	4p16	LD, M	Van der Boogaard et al., 2000; Maestri et al., 1997; Mitchell et al., 2001; Romitti et al., 1999; Jezewski, 2003; Lidral et al., 1998; Vieira et al., 2003; Suzuki et al., 2004.
FOXE1	9q22	L, LD, M	Vieira et al., 2005b; Venza et al., 2006; Moreno et al., 2009a.
FGFR2	10q26	M	Riley et al., 2007; Riley and Murray, 2007; Osoegawa, 2008.
BMP4	14q22–q23	M	Lin et al., 2008; Suzuki et al., 2009; Jianyan et al., 2010.
	17q22 locus	GWA	Beaty et al., 2010; Mangold et al., 2010.
MAFB	20q12	GWA	Beaty et al., 2010.
MYH9	20q13	LD	Birnbaum et al., 2009; Martinelli et al., 2007; Chiquet et al., 2009; Jia et al., 2010.

Gene	Location	Evidence	References
Intensively studied			
MTHFR	1p36.3	LD	Martinelli et al., 2001; Blanton et al., 2000; Botto and Yang, 2000; Jugessur et al., 2003b; Jagomägi et al., 2010.
TGF-α	2p13	LD	Marazita and Mooney, 2004; Mitchell, 1997; Hwang et al.,1995; Maestri et al.,1997; Miettinen et al., 1989; Suzuki et al., 2004; Carter et al., 2010.
SUMO1	2q33	M	Alkuraya et al., 2006; Shi et al., 2009; Mostowska et al., 2010; Carter et al., 2010.
PDGFC	4q32	LD, M	Ding et al., 2004; Choi et al., 2009; Jugessur et al., 2009.
FGF8	10q24	M	Riley and Murray, 2007; Riley et al., 2007.
PVRL1	11q23	M, LD	Avila et al., 2006; Sözen et al., 2001; Sözen et al., 2009.
TGF-β3	14q24	LD, M	Miettinen et al.,1999; Maestri et al.,1997; Hwang, 1992; Lidral et al.,1998; Suzuki et al., 2004; Beaty et al., 2002; Suazo et al., 2010.
CRISPLD2	16q24	LD	Chiquet et al., 2007; Letra et al., 2010
GSTT1	22q11	LD	Shi et al., 2007

GWA = genome-wide association; LD = linkage disequilibrium; L = linkage; M = mutation detection

10.2.1. Chromosome 1

10.2.1.1. *IRF6* – interferon regulatory factor 6; 1q32.3–q41

IRF6 belongs to a family of nine transcription factors which regulate the expression of interferon-alpha and interferon-beta after viral infection. Zucchero et al. (2004) found evidence for overtransmission of several single nucleotide polymorphisms (SNPs) in *IRF6* in nonsyndromic CLP, several of which were confirmed by others (Blanton et al., 2005; Scapoli et al., 2005; Diercks et al., 2009; Jia et al., 2009).

Mutations in the *IRF6* gene are known to be associated with van der Woude syndrome and popliteal pterygium syndrome. Variation at the *IRF6* locus is responsible for 12% of the genetic contribution to CL/P at the population level and triples the recurrence risk for a child with a cleft in some families (Houdayer et al., 2001; Zucchero et al., 2004; Scapoli et al., 2005).

Although the role of *IRF6* during embryonic development has been identified, its function and regulation still remain poorly understood. *IRF6* belongs to the IRF family of transcription factors. This gene encodes interferon

regulatory factor 6, which is a key element in oral and maxillofacial development. *IRF6* is highly expressed in the leading edge ectoderm of the palatal shelves before and during formation of the primary palate (Knight et al., 2006; Washbourne and Cox, 2006). It has been shown that *IRF6*-null mice have abnormal skin, limb, and craniofacial development (Kondo et al., 2002).

A positive association between *IRF6* variants and OC has been confirmed in multiple populations and independently replicated (Vieira et al., 2007a). Meta-analysis of 13 genome scans confirmed that *IRF6* is one of the main candidate genes that has common polymorphic variants, which can increase the risk of CL/P (Marazita et al., 2004).

Further functional analyses to identify downstream target genes and interacting proteins is important to the understanding of the role of *IRF6* in palatal development, especially given (1) the overlap of *IRF6* gene expression at the medial edge of the palatal shelves immediately before and during fusion with that of transforming growth factor beta 3 (*TGF-β3)* in mice, and (2) the proposed role of the SMIR domain of *IRF6* in mediating interactions between IRFs and Smads, a family of transcription factors known to transduce *TGF-β* signals (Fitzpatrick et al., 1990; Brivanlou & Darnell, 2002).

It has been shown that integration of *IRF6* and the Notch ligand Jagged2 function is essential for the control of palatal adhesion and fusion competence via a combined role in the control of oral periderm formation and differentiation (Richardson et al., 2009).

Van der Woude syndrome (VWS).

VWS represents the most common single-gene cause of cleft lip and cleft palate, accounting for about 2% of all individuals with CL/P (Cohen and Bankier, 1991; Murray et al., 1997) or roughly one in 35,000 to one in 100,000 in the European and Asian populations (Cervenka et al., 1967; Rintala and Ranta, 1981; Burdick, 1986).

Patients with VWS have clefts of the lip and palate, missing teeth in approximately 25% of cases, and pits in the lower lip in approximately 85% of cases. Both cleft types, CL/P and CP only, occur in individuals with VWS in the same proportions as in the general population, about two to one respectively (Burdick et al., 1987). Oberoi and Vargervik (2005) suggest that individuals with VWS are more likely to have hypoplasia of the mandible and maxilla than isolated cases with the same cleft phenotype.

Sequence analysis of the *IRF6* coding region (exons 1 through 9) detects mutations in approximately 70% of individuals with VWS. Mutations in exons 3, 4, and 7–9 account for 80% of known VWS-causing mutations (Shutte and Murray, 1999).

Popliteal pterygium syndrome (PPS).

Prevalence is approximately one in 300,000. The PPS phenotype includes CL/P in approximately 91–97% of individuals; fistulae of the lower lip in 45% of cases (Froster-Iskenius, 1990); webbing of the skin extending from the ischial

tuberosities to the heels, bifid scrotum and cryptorchidism in males, hypoplasia of the labia majora in females, syndactyly of fingers and/or toes, and anomalies of the skin around the nails (Rintala and Lahti, 1970).

Most missense mutations that cause PPS are located in *IRF6* exon 4. It appears likely that certain mutations (R84H, R84C) are more apt to cause PPS than VWS. A cluster of missense mutations in the DNA-binding domain are more commonly seen in families with PPS. However, families may include individuals with features of only VWS, and other members with the additional features of PPS.

10.2.1.2. *MTHFR* – methylenetetrahydrofolate reductase; 1p36.3

MTHFR is an important enzyme in folate metabolism. The *MTHFR* gene encodes an enzyme called methylenetetrahydrofolate reductase. This enzyme plays a role in processing amino acids, the building blocks of proteins. Methylenetetrahydrofolate reductase is important for a chemical reaction involving forms of the vitamin folate (also called folic acid or vitamin B9). Specifically, this enzyme converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. This reaction is required for the multistep process that converts the amino acid homocysteine to another amino acid, methionine. The body uses methionine to make proteins and other important compounds.

In 1998, Shaw reported an association between CL/P and genetic variation at the *MTHFR* locus. Since that initial report, there have been a number of studies reporting the association between CL/P and *MTHFR* variant (Mills et al., 1999; Martinelli et al., 2001). The gene encoding the *MTHFR* enzyme is known to have at least two functional polymorphisms: 677 C>T (rs1801133, c.665C>T, p. Ala222Val) and 1298 A>C (rs1801131, c.1286A>C, p. Glu429Ala). The homozygous *MTHFR 677TT* genotype results in a thermolabile enzyme with reduced activity (Molloy et al., 1997). A second polymorphism in the *MTHFR* gene, an *A*-to-*C* substitution at nucleotide 1298, also results in decreased *MTHFR* activity but is not associated with higher homocysteine or lower plasma folate levels (Van der Put, 1998). Animal studies suggest that a decreased conversion of homocysteine to methionine could be a crucial step in causing neural tube defects. It has been shown that rat embryos in culture require methionine for neural tube closure (Mills et al., 1996). Several case-control studies have attempted to implicate this polymorphism in clefting etiology but results have not been encouraging. Associations have only been found in small studies (Shaw et al., 1998; Gaspar et al., 1999).

10.2.2. Chromosome 2

10.2.2.1. *TGF-α* – Transforming growth factor alpha; 2p13

Transforming growth factors (TGFs) are biologically active polypeptides that reversibly confer the transformed phenotype on cultured cells.

The *TGF-α* receptor is identical to the epidermal growth factor (EGF) receptor. *TGF-α* shows about 40% sequence homology with EGF and competes with EGF for binding to the EGF receptor, stimulating its phosphorylation and producing a mitogenic response.

The biological activities of *TGF-α* resemble those of EGF since both factors bind to the same receptor. Some biological activities of *TGF-α* are, however, stronger than those of EGF. *TGF-α* is thought to be the fetal form of EGF. The physiological role of *TGF-α* is probably the control of epidermal development during development and differentiation of the cells (Ardinger et al., 1989). *TGF-α* also affects bone formation and remodelling by inhibition of the synthesis of collagen and release of calcium. These effects are more pronounced than those of EGF. *TGF-α* also promotes the generation of osteoblast-like cells in long-term bone marrow cultures.

The first study showing association of *TGF-α* with CL/P was by Ardinger in 1989, and many additional studies have replicated this finding (Marazita and Mooney, 2004; Mitchell, 1997; Hwang et al., 1995; Maestri et al.,1997; Miettinen et al., 1989; Suzuki et al., 2004; Carter et al., 2010). However, other studies have not been able to replicate this finding by either linkage or association (Jugessur et al., 2003a; Passos-Bueno, 2004). A study combining 13 linkage scan studies (Marazita et al., 2004), revealed positive results, corroborating the hypothesis that *TGF-α* is a modifier rather than being necessary or sufficient to cause clefting.

Chromosomal abnormalities involving only duplication of chromosome 2q are rare. Duplication within the long arm of chromosome 2 may cause CPO and Pierre Robin sequence (Õunap et a., 2005).

10.2.3. Chromosome 4

10.2.3.1. *MSX1* – msh homeobox; 4p16.3–p16.1

MSX genes are homeobox-containing genes homologous to the *Drosophila msh* gene (Hill et al., 1989). *MSX* proteins function as transcriptional repressors in cellular differentiation (Catron et al., 1996) and interact with other protein factors to modulate differentiation and proliferation (Zhang et al., 1997). Embryonic expression patterns of *MSX* genes are consistent with the role of *Msx* proteins in epithelial-mesenchymal tissue interactions during craniofacial development (Hill et al., 1989). The role of *Msx* proteins in active morphogenesis is suggested by the lack of *Msx1* expression in cells undergoing terminal differentiation (Woloshin et al., 1995) and by restricted cellular expression of *Msx1* transcript during periods of rapid cellular proliferation

(Simon et al., 1995). Point mutations in *MSX1* appear to contribute to approximately 2% of all CL/P cases (Jezewski et al., 2003). *Msx1*-deficient mice develop craniofacial abnormalities of the nasal, frontal, and parietal bones, as well as CP. The occurrence of CP in *Msx1* knockout mice aided the identification of a *MSX1* mutation cosegregating with tooth agenesis, CL/P and CP (van den Boogaard et al., 2000). It has been proposed that CP in *Msx1* knockout mice is due to insufficient palatal mesenchyme (Ferguson, 1994). Also, rare human mutations have been observed in *MSX1* that are associated with tooth agenesis (Vastardis et al., 1996), with and without CL/P (van den Boogaard et al., 2000). Association and linkage studies further support a role for *MSX1* in different populations.

10.2.4. Chromosome 6

10.2.4.1. *EDN1* – endothelin-1; 6p24.1

The protein encoded by this gene is proteolytically processed to release a secreted peptide termed endothelin-1. This peptide is a potent vasoconstrictor and is produced by vascular endothelial cells. *EDN1* also can affect the central nervous system.

Endothelin receptors are widely expressed in all tissues, which is consistent with their physiological role as vasoactive peptides. They are also localized to non-vascular structures including epithelial cells, glia and neurons. The principle physiological role of endothelin receptors is the maintenance of vascular tone.

Knockout mice of *EDN1*, which is homologous to *EDN1* mapping to the chromosomal region 6p23 in humans, shows craniofacial abnormalities including cleft palate (Kurihara et al., 1994). Pezzetti et al. (2000) examined the endothelin gene and three other genes in the endothelin pathway (*ECE1, EDNRA, EDNRB*) as possible candidates for orofacial cleft. Linkage results indicated that none of these genes is involved in the pathogenesis of OC. Most of the studies have excluded the possibility that the *EDN1* pathway plays a major role in the etiology of nonsyndromic CL/P in humans (Scapoli et al., 2002), but several studies have shown some evidence of CL/P locus on the 6p23–25 regions. Linkage has been found with *EDN1* and *AP2* genes, and balanced translocations and deletions in this region associated with a cleft phenotype have been reported (Moreno et al., 2004).

10.2.5. Chromosome 9

10.2.5.1. *FOXE1* – forkhead box E1 (thyroid transcription factor 2); 9q22

Mutations in *FOXE1* are associated with congenital hypothyroidism, thyroid agenesis and CP in humans (Bamford-Lazarus syndrome, MIM 241850) and mice (De Felice et al., 1998; Dathan et al., 2002). The forkhead gene family

(*FOX*), originally identified in *Drosophila*, encodes transcription factors with a conserved 100-amino acid DNA-binding motif called the 'forkhead domain' and regulates diverse developmental processes in eukaryotes. Rare missense mutations in *FOXE1* have been associated with isolated clefting (Vieira et al., 2005b; Venza et al., 2006).

The study by Marazita et al. (2009) showed the strongest associations with families in which one or more affected family members had CL/P, and little or no evidence of association in families with CL alone or CP alone. In a cohort of CL/P families from Colombia, the United States, and the Philippines, Moreno et al. (2009a) tested 397 SNPs spanning 9q22-q33 for association. Significant SNP and haplotype association signals narrowed the interval to a 200-kb region containing *FOXE1*, C9orf156, and *HEMGN*. Association results were replicated in CL/P families of European descent; when all populations were combined, the two most associated SNPs, rs3758249 (P = 5.01E^{-13}) and rs4460498 (P = 6.51E^{-12}), were located inside a 70-kb linkage disequilibrium block containing *FOXE1*. Isolated cleft palate was also associated, indicating that *FOXE1* may play a role in two phenotypes thought to be genetically distinct.

The involvement of *FOXE1* during primary palatogenesis is supported by the previously uncharacterized epithelial expression in the medial nasal and maxillary processes that will undergo fusion (Marazita et al., 2009).

10.2.6. Chromosome 14

10.2.6.1. *TGF-β3* – transforming growth factor, beta 3; 14q24

Transforming growth factor betas (*TGF-β*) mediate many cell-cell interactions that occur during embryonic development. *TGF-β* exists in at least five isoforms, known as *TGF-β1, TGF-β2, TGF-β3, TGF-β4, TGF-β5*, that are not related to TGF-alpha. Their amino acid sequences display homologies on the order of 70–80%. A *Tgf-β3* knockout mouse with defective palatogenesis was present by Kaartinen (1995). Proetzel et al. (1995) produced *Tgf-β*-null mice in which exon 6 of the *TGF-β3* gene was replaced by the neomycin-resistance gene. Whereas heterozygotes had no apparent phenotypic change, homozygotes had an incompletely penetrant failure of the palatal shelves to fuse, leading to CP. The defect appeared to result from impaired adhesion of the apposing medial edge epithelial of the palatal shelves and subsequent elimination of the midline epithelial seam. Subsequent human studies have yielded both positive and negative results (Marazita and Mooney, 2004).

10.2.6.2. *JAG2* – Protein jagged-2 precursor; 14q32

The Notch family of receptors are important signalling molecules regulating cell fate during development. Jagged 1 and Jagged 2 proteins play a role in craniofacial and limb development. Targeted deletion of the *JAG2* exons encoding the DSL domain results in craniofacial defects and perinatal lethality in mice (Jiang et al., 1998). *Jag2* is expressed throughout the oral epithelium

and is required for Notch1 activation during oral periderm differentiation. The mutant homozygotes exhibited CP and fusion of the tongue with the palatal shelves. *Jag2* mutant mice have CP mainly due to failure of the palatal shelves to elevate and fuse.

Richardson et al. (2009) showed that *Irf6 /Jag2* doubly heterozygous mice displayed fully penetrant intraoral epithelial adhesions, resulting in CP. There was no evidence of direct interaction between *Irf6* and *Jag2*, suggesting that the mechanism underlying the genetic interaction between *Irf6* and *Jag2* is the consequence of their combined effects on periderm formation, maintenance, and function.

10.2.7. Chromosome 17

10.2.7.1. *RARA* – retinoic acid receptor, alpha; 17q21

The *RARA* gene was first reported by Bale et al. (1988). Mattei et al. (1991) mapped the *RARA* genes in humans, mice, and rats, thereby extending the homologies among human chromosome 17, mouse chromosome 11, and rat chromosome 10. Juriloff and Mah (1995) studied A/WySn-strain mice with a high birth prevalence of CL/P, an animal model with a similarly complex genetic basis. They mapped a major CL/P-causing gene, *clf1*, to chromosome 11 to a region having linkage homology with humans.

Retinoic acid has a well-established role during development, and members of the retinoic acid receptor family mediate its activity. Transgenic and knockout mice studies have shown that these genes are important for facial development (Lohnes et al., 1994). Various human studies have reported both positive and negative results near the *RARA* gene. Chenevix-Trench et al. (1992) first reported a significant difference in the frequency of alleles at the *RARA* locus between nonsyndromic CL/P patients and unrelated controls. Vintiner et al. (1993) investigated a group of British CL/P samples and found no association or linkage between *RARA* and the traits. Shaw et al. (1993) performed linkage analyses on 14 Indian families in West Bengal, India. They also reported no linkage between nonsyndromic OC and *RARA*.

10.2.8. Chromosome 19

10.2.8.1. *PVRL2* – Poliovirus receptor-related 2 (herpes virus entry mediator B); 19q13.2

PVRL2 is a transmembrane glycoprotein that belongs to the poliovirus receptor family. Mutations in a related protein, *PVRL1*, are known to cause the autosomal recessive Margarita Island clefting syndrome (Suzuki et al., 2000).

This gene encodes a single-pass type I membrane glycoprotein with two Ig-like C2-type domains and an Ig-like V-type domain. This protein is one of the plasma membrane components of adherent junctions. It also serves as an entry

for certain mutant strains of herpes simplex virus and pseudorabies virus, and it is involved in cell-to-cell spreading of these viruses. Variations in this gene have been associated with differences in the severity of multiple sclerosis. Alternate transcriptional splice variants, encoding different isoforms, have been characterized (Suzuki et al., 2000).

10.2.8.2. *BCL3* – B-cell CLL/lymphoma 3; 19q13.1–q13.2

BCL3 is a protooncogen that is involved in cell proliferation, differentiation and apoptosis. Previous evidence has implicated the role of the *BCL3* gene in the etiology of nonsyndromic clefting. Several studies have observed an association between *BCL3* alleles and OC, and the association has been suggested to be due to either an allele of low penetrance or *BCL3* acting as a modifier locus (Maestri et al., 1997; Gaspar et al., 2002).

10.2.9. Chromosome X

10.2.9.1. *TBX22* – T-box 22 T-box transcription factor; Xq21.1

This gene is a member of a phylogenetically conserved family of genes that share a common DNA-binding domain, the T-box. T-box genes encode transcription factors involved in the regulation of developmental processes. Braybrook et al. (2001) identified six different mutations, including missense, splice site, and nonsense, in the *TBX22* gene in families segregating X-linked disorder cleft palate with ankyloglossia (CPX), and it is believed to play a major role in human palatogenesis.

Marcano et al. (2004) analysed the *TBX22* gene in a large sample of patients with CP with no preselection for inheritance or ankyloglossia. They found *TBX22* coding mutations in 5 of 200 patients in North American and Brazilian cohorts, with an additional four putative splice site mutations. They also identified mutations in previously unreported CPX families and presented a combined genotype/phenotype analysis of previously reported familial cases. Males frequently exhibited CP and ankyloglossia together (78%), as did a smaller percentage of carrier females. Mutations within families could result in either CP only, ankyloglossia only, or both, indicating that these defects are distinct parts of the phenotypic spectrum.

Andreou et al. (2007) suggested that small ubiquitin-related modifier (SUMO) modification may represent a common pathway that regulates normal craniofacial development and is involved in the pathogenesis of orofacial clefting. He found that *TBX22* is a target for *SUMO1* and that this modification is required for *TBX22* repressor activity. *SUMO1* haploinsufficiency leads to OC (Alkuraya, 2006). Although the site of SUMO attachment at lysine-63 is upstream of the T-box domain, loss of *SUMO1* modification is consistently found in all pathogenic X-linked CP missense mutations. This implies a general mechanism linking the loss of SUMO conjugation to the loss of *TBX22* function.

AIMS OF THE STUDY

1. To evaluate the occurrence rate of OC, on the basis of records of patients treated in the Department of Oral and Maxillofacial Surgery of the Tartu University Hospital, during the period of 1910–2000.
2. To determine the rate of occurrence between different cleft types on the basis of gender and location.
3. To record the epidemiological factors which may influence the development of OC, and to evaluate their occurrence regularities.
4. To investigate the possible contribution of recognized candidate genes in the development of nonsyndromic OC in an Estonian population and in the Baltic region (Estonia, Latvia, Lithuania).

MATERIALS AND METHODS

1. Study population

For the purpose of the study I (Ref. I), data was collected from the preserved database in the Department of Oral and Maxillofacial Surgery at the Stomatology Clinic of Tartu University Hospital. The preserved patient records were available for the years 1910 to 2000. During this period of time, a total of 585 health files of patients with OC had been preserved. In 583 cases, the patient's gender was known (this information was missing in two files).

Table 4. Overview of the cohorts and genetic markers used in the present study

Ref. number	Sample size	Cleft sample	Controls	Sample origin	Candidate genes	Genotyped SNPs
I	583[1] F= 251 M=332	CLP 245 CP 227 CL 111	–	Estonia	–	–
II	358	153[1] F= 79; M=74 CL/P 100 CP 53	205[4]	Estonia	CLP 18 CP 40	176
III	710	104[1,2,3] F= 60 M=44	606[4,5,6] Estonia 205 Latvia 182 Lithuania 219 F= 338 M=268	Estonia, Latvia, Lithuania	40	591
IV	906	300[1,2,3] CL 66 CL/P 234 Estonia 100[1] Latvia 108[2] Lithuania 92[3]	606[4,5,6] Estonia 205[4] Latvia 182[5] Lithuania 219[6] F= 338 M=268	Estonia, Latvia, Lithuania	40	587

1. Patients were recruited from surgical clinics in Estonia (North Estonia Medical Centre, Tallinn, and Tartu University Hospital).
2. Patients were collected at the Riga Cleft Lip and Palate Centre, Institute of Stomatology, Riga Stradins University.
3. Patients were collected at the Center for Medical Genetics, Vilnius University Hospital Santariškių Klinikos in collaboration with the largest orthodontic clinics in Lithuania.
4. Unaffected unrelated individuals without a family history of clefting, collected as randomly selected population-based controls from a Biobank of the Estonian Genome Center, University of Tartu.
5. Controls selected from the Latvian Biomedical Research and Study Center within the framework of the national project "Genome Database of Latvian Population".
6. Control sample of Lithuania consists of unrelated individuals who were recruited from six different ethnolinguistic groups (i.e., East Aukštaičiai, West Aukštaičiai, South Aukštaičiai, West Žemaičiai, South Žemaičiai and North Žemaičiai) with an equal male-to-female ratio.

All probands from the Lithuanian and Latvian sample from candidate gene studies (Ref. III and IV) were identified by an experienced clinical geneticist for congenital anomalies or major developmental delays. In the Estonian sample (Ref. I, II, III and IV) diagnostic information from clinical geneticists was available in patient medical records. Patients with confirmed monogenic syndrome or chromosomal aberrations, associated malformations and mental retardation were excluded from the study.

Ethical approval for the study was obtained from the Ethics Review Committee on Human Research of the University of Tartu, the central Medical Ethics Committee of Latvia, and the Lithuanian Bioethics Committee. All individuals signed an informed consent form for participation in the study. In the case of patients who were under18 years of age, consent was obtained from their parent.

2. Methods

2.1. Genes and SNP selection

We selected candidate genes on the basis of previously published findings from association and linkage studies of Caucasian origin on nonsyndromic OC, gene expression patterns during craniofacial development, gene-knockout data from animal studies, genes that underlie Mendelian syndromic forms of clefting, and studies of chromosomal rearrangements associated with cleft phenotypes in humans. Selected SNPs were selected to capture all the SNPs with minor allele frequencies $\geq$0.05 and $r^2 \geq 0.8$ in the regions of interest based on the HapMap Phase II data, using HapMap CEU as a reference population. Multiple SNPs were selected for each gene, including 10–20 kb of both upstream and downstream genomic sequences. A list of selected genes in different studies and the number of genotyped SNPs per gene are shown in Table 5.

Table 5. Candidate genes and loci included in the study

Gene	Chromosome	Study II Genotyped SNPs	Study III Genotyped SNPs	Study IV Genotyped SNPs
MTHFR	1p36.3	11	9	8
LHX8	1p31.1	9	9	9
COL11A1	1p21	–	42	42
SKI	1q22-q24	20	19	19
IRF6	1q32.3-q41	11	10	10
TGF-α	2p13	–	36	35
FN1	2q34	–	27	27
MSX1	4p16.3-p16.1	15	15	15
FGF2	4q26-q27	–	18	18
FGF1	5q31	–	31	31
MSX2	5q34-q35	6	6	6

Gene	Chromosome	Study II Genotyped SNPs	Study III Genotyped SNPs	Study IV Genotyped SNPs
EDN1	6p24.1	15	15	15
COL11A2	6p21.3	–	19	19
FGFR1	8p11.2-p11.1	–	11	11
FOXE1	9q22	4	4	4
TBX10	11q13.2	10	10	10
MMP3	11q22.3	–	5	5
MMP13	11q22.3	–	19	19
PVRL1	11q23.3	18	17	17
COL2A1	12q13.11	–	32	32
SPRY2	13q31.1	3	–	–
BMP4	14q22-q23	–	4	4
TGF-β3	14q24	8	8	8
JAG2	14q32	11	11	11
MMP25	16p13.3	–	6	6
MMP2	16q13-q21	–	20	20
CDH1	16q22.1	–	13	13
RARA	17q21	5	4	4
WNT3	17q21	–	16	16
WNT9B	17q21	–	11	11
TIMP2	17q25	–	25	25
'OFC11'	18q21[a]	–	25	25
BCL3	19q13.1-q13.2	4	3	2
PVRL2	19q13.2	13	12	12
CLPTM1	19q13.2-q13.3[b]	8	7	7
BMP2	20p12	–	25	25
MMP9	20q11.2-q13.1	–	6	5
TIMP3	22q12.3	–	36	36
TBX22	Xq21.1	5	5	5

[a] includes *SMAD2* and *SMAD4* genes
[b] includes *APOC2* gene

2.2. Genotyping

A sample of peripheral venous blood was taken from all participants, for DNA extraction. Genomic DNA was extracted from EDTA-preserved blood according to standard high-salt extraction (Estonian sample) or phenol-chloroform methods (Latvian and Lithuanian samples). SNP genotyping was performed using an arrayed primer extension-based genotyping method (APEX-2). This method allows multiplex DNA amplification and detection of SNPs, and mutations on microarrays via four-colour single-base primer extension (Krjutškov et al., 2008).

3. Statistical analysis

Descriptive statistics were performed using the Statistical Package for the Social Sciences (SPSS) version 14.0 software package. All markers were assessed for Hardy–Weinberg equilibrium in controls and affected individuals using a Pearson's chi-square test with one degree of freedom. The alleles at each marker were tested for association, twice: first, only individuals with CL/P were compared with controls; and, second, only individuals with CP were compared with controls. Allelic odds ratios (ORs) and 95% confidence intervals (CIs) were estimated using the standard chi-square test, assuming a multiplicative model. The level of statistical significance was set at $\alpha = 0.05$ for nominal association without correction for multiple comparisons because of the exploratory character of this study. Haplotype-phenotype association tests were performed using the standard chi-square test. Statistical analyses were conducted using PLINK 1.06 software (Purcell et al., 2007).

RESULTS

1. Overview of Estonian OC patients

1.1. Study group outline

During the period 1910–2000, a total of 585 health files of patients with OCs had been preserved. In 583 cases, the patient's gender was known (this information was missing in two files); there were 333 boys (57.1%) and 250 girls (42.9%) (Figure 6).

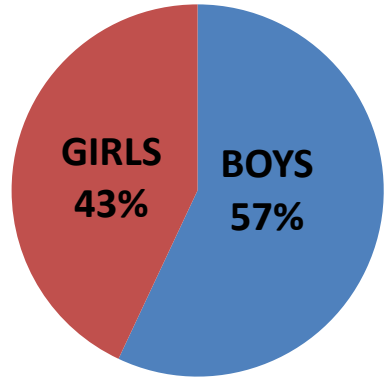


Figure 6. Gender of the patients

Forty two percent of clefts were CLP, 19% were CL and 39% were CP (Figure 7). One of the findings of the study, was that there was a high occurrence rate of CP: CL (19%), CLP (42%), CP (39%) – a ratio of 1:2:2.

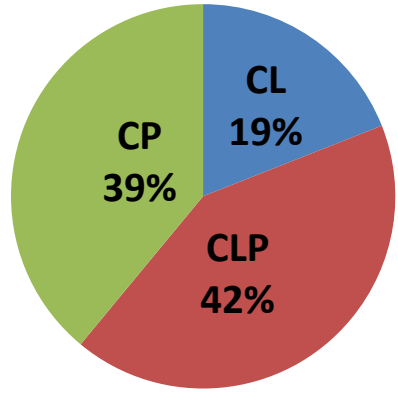


Figure 7. Occurrence of different cleft types

The most common cleft type was incomplete cleft palate (30% of patients with clefts), the least frequent was bilateral cleft lip (3.8%). The most common cleft type for boys was left-side CLP (13.8%) and the most common for girls was CP (17.8%). The least frequently occurring cleft was bilateral CL: boys (2.6%) and girls (1.2%) (Figure 8). The left side of the face was affected 2.2 times more frequently than the right side.

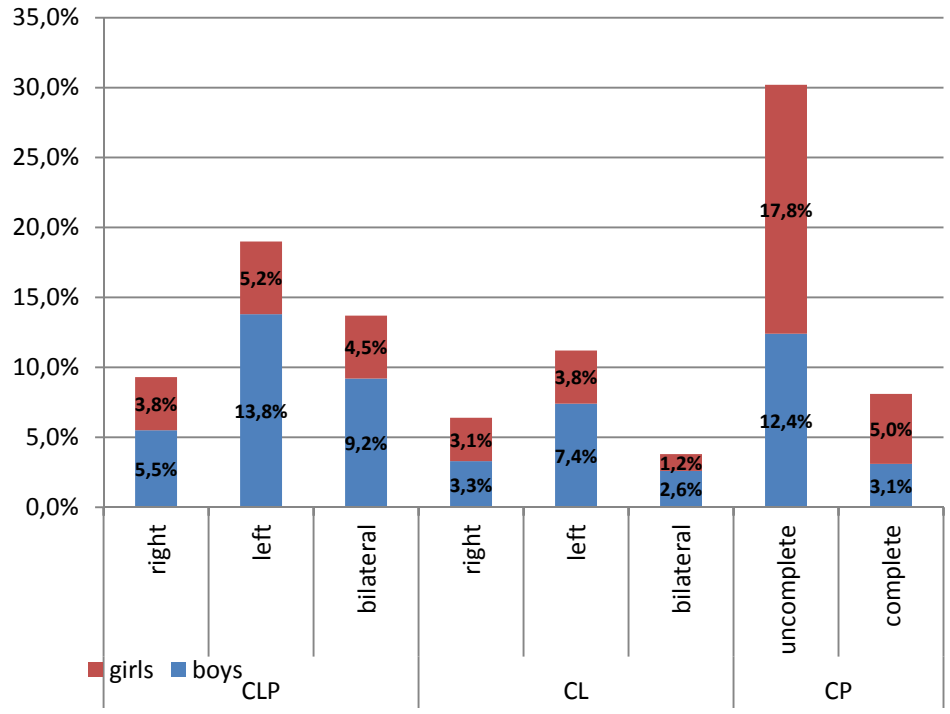


Figure 8. The most common cleft types according to gender and facial involvement.

1.2. Epidemiological factors

Four hundred and eighty-eight patients with clefts also had their birthweight marked on their patient records. Of these, 2.6% were born prematurely. The average birthweight of children with clefts was 3416 grams (boys 3447 g, girls 3376 g), and 6.8% had a birthweight of less than 2500 g. Half of the children had developmental anomalies.

In terms of age, 28.4% of mothers and 37.7% of fathers were older than 30 years; both parents were older than 30 years in 21.9% of cases; 2.6% of mothers were older than 40 years; and 53% of subjects were between the ages of 20 and 30.

Epidemiological factors which affected the mother in the first trimester of pregnancy (Figure 9) included:

* Physical factors – 5.8% had physical traumas, 12.9% did heavy physical labour and 45% underwent medical abortions before the pregnancy.
* Chemical factors – 6.7% had toxicosis during the first trimester, 5.2% had hormonal dysbalance, 5.2% had exposure to chemicals.
* Biological factors – 9.8% had a common cold, 4% had gynaecological disorders.
* Psychological factors – 36 % reported stress or fright.

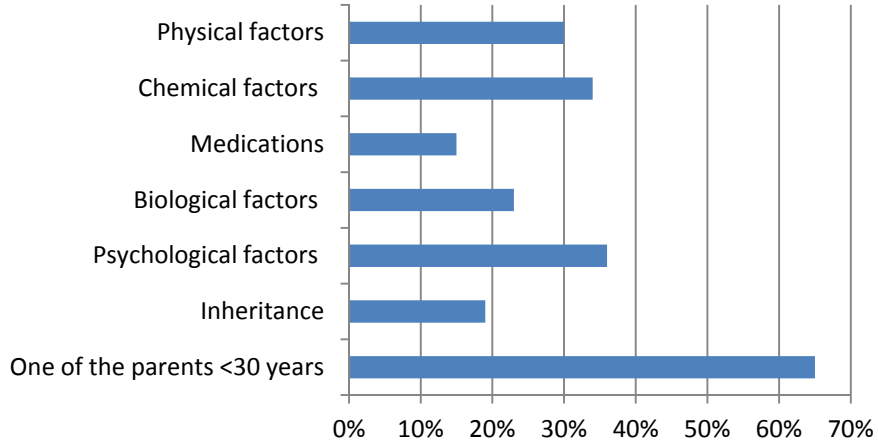


Figure 9. Epidemiological factors which affected the mother in the first trimester of pregnancy

2. Genes contributing to the risk of nonsyndromic CL/P in Estonia

We genotyped 176 tag SNPs in 18 candidate genes in 100 CL/P patients and 205 unrelated controls from the Estonian population. The overall call rate was 99.62%. There was no significant deviation from the Hardy-Weinberg equilibrium for any of the genotyped SNPs.

Table 6 presents all markers with allelic association test *P*-values of <0.05 in the CL/P case-control sample. Twenty-six polymorphisms in 10 genes, and 9 out of 26 SNPs, displayed nominal evidence of association with the CL/P phenotype.

Table 6. Most significant results from single-marker association analysis of the CL/P group

Gene	Chr	SNP	Location	Alleles[a]	MAF [b]		*P*	OR	95% CI
					Cases	Controls			
MTHFR	1	rs1476413	11774887	C/T	0.235	0.344	0.0062	0.586	0.399–0.861
		rs1801131	11777063	T/G	0.250	0.360	0.0067	0.594	0.406–0.867
		rs1994798	11777342	A/G	0.320	0.446	0.0028	0.584	0.409–0.833
		rs4846054	11791817	T/C	0.320	0.434	0.0070	0.614	0.430–0.877
		rs2076001	11801854	A/G	0.075	0.139	0.0214	0.502	0.277–0.911
IRF6	1	rs590223	208013330	A/G	0.435	0.349	0.0392	1.438	1.018–2.031
SKI	1	rs1809822	2221455	A/C	0.040	0.107	0.0057	0.350	0.162–0.759
MSX1	4	rs11726039	4900371	T/C	0.207	0.283	0.0452	0.662	0.441–0.993
		rs868257	4903074	C/G	0.375	0.281	0.0180	1.539	1.076–2.202
		rs6446693	4905981	C/T	0.542	0.379	0.000182	1.934	1.366–2.738
		rs1907998	4907480	A/G	0.450	0.337	0.0066	1.613	1.141–2.279
		rs6832405	4934637	G/T	0.222	0.304	0.0351	0.654	0.440–0.972
EDN1	6	rs6912834	12403521	A/G	0.175	0.113	0.0358	1.660	1.031–2.674
		rs4714384	12405839	T/C	0.412	0.307	0.0120	1.576	1.104–2.249
		rs6906760	12409657	A/T	0.195	0.127	0.0265	1.668	1.058–2.628
		rs9471438	12415250	T/C	0.480	0.373	0.0117	1.551	1.101–2.183
TBX10	11	rs2509713	67165367	C/T	0.220	0.151	0.0353	1.583	1.030–2.434
TGF-β3	14	rs2205181	75523872	T/C	0.390	0.307	0.0422	1.441	1.012–2.052
		rs8011635	75534584	C/T	0.374	0.290	0.0382	1.459	1.020–2.088
JAG2	14	rs11624283	104662016	A/G	0.095	0.154	0.0462	0.578	0.336–0.996
		rs2056860	104666863	C/T	0.255	0.185	0.0468	1.504	1.004–2.253
		rs1022431	104674460	C/A	0.125	0.063	0.0099	2.110	1.184–3.758
BCL3	19	rs8100239	49944944	T/A	0.245	0.337	0.0249	0.638	0.430–0.946
		rs8103315	49946008	C/A	0.190	0.117	0.0151	1.769	1.112–2.814
PVRL2	19	rs519113	50068124	C/G	0.185	0.271	0.0204	0.612	0.403–0.929
		rs2075642	50069307	G/A	0.235	0.139	0.0031	1.902	1.237–2.925

[a] Major allele (in controls) is listed first

[b] MAF = minor allele frequency

The most significant association with CL/P was found for SNP rs6446693, which is located ~6 kb upstream of the muscle segment homeobox 1 (*MSX1*) gene, and this association remained statistically significant after correcting for multiple testing. The T allele was associated with significantly higher risk (OR = 1.934; 95% CI = 1.366–2.738; $P = 1.82 \times 10^{-4}$). The SNP rs1907998, located ~5 kb upstream of *MSX1*, was significantly associated with CL/P under the assumption of the dominant model of inheritance ($P = 8.53 \times 10^{-5}$).

Other markers with interesting P-values in the CL/P dataset included the *MTHFR, SKI*, *MSX1*, and *PVRL2* loci (Table 6). Among markers in or near the *MTHFR* and *SKI* genes, a variant allele had a protective effect, whereas the variant alleles of the *MSX1* and *PVRL2* markers were associated with an increased risk.

There was no evidence of a sex-specific component in the association between CL/P and the nine markers with the best *P*-values. The genotype distributions in male and female cases were similar for these SNPs (Armitage's trend test $P > 0.05$).

Haplotype analysis

Haplotype analysis was performed only with the CL/P dataset (cases plus controls, N = 305). Case-control analysis was performed as single haplotype comparisons for haplotypes constructed from SNPs within linkage disequilibrium (LD) blocks identified in the *MSX1, MTHFR, IRF6, BCL3, TGF-β3, PVRL2, EDN1, JAG2,* and *TBX10* genes.

Table 7. Haplotype-phenotype association analysis of the *MTHFR* gene

	SNP1	SNP2	SNP3	SNP4	SNP5	Frequency		
Haplotype	rs1476413	rs1801131	rs1994798	rs4846054	rs17376328	Cases	Controls	*P*
H1	C	T	A	T	G	0.639	0.531	0.0114
H1	*	T	A	T	G	0.660	0.541	0.0053
H1	*	*	A	T	G	0.660	0.544	0.0063
H2	T	G	G	C	*	0.215	0.324	0.0053
H1	C	T	A	*	*	0.659	0.536	0.0038
H2	T	G	G	*	*	0.214	0.326	0.0044
H1	*	T	A	T	*	0.660	0.541	0.0053
H2	*	G	G	C	*	0.235	0.342	0.0069
H1	*	*	*	T	G	0.680	0.567	0.0072
H1	C	T	*	*	*	0.730	0.629	0.0133
H2	T	G	*	*	*	0.215	0.331	0.0030
H1	*	*	A	T	*	0.660	0.544	0.0063
H2	*	*	G	C	*	0.300	0.424	0.0030
H1	*	T	A	*	*	0.680	0.546	0.0016
H2	*	G	G	*	*	0.250	0.352	0.0106

	SNP1	SNP2	SNP3	SNP4	SNP5	Frequency		
Haplo-type	rs1476413	rs1801131	rs1994798	rs4846054	rs17376328	Cases	Controls	*P*
H1	C	*	A	*	*	0.658	0.537	0.0045
H2	T	*	G	*	*	0.213	0.327	0.0036
H1	C	*	*	T	*	0.659	0.554	0.0136
H2	T	*	*	C	*	0.214	0.330	0.0029
H1	*	T	*	T	*	0.664	0.553	0.0089
H2	*	G	*	C	*	0.234	0.346	0.0048

Table 7 presents the results from haplotype-based association analysis within an LD block at the *MTHFR* locus for all possible 2- to 5-SNP haplotypes with a frequency of > 5% among CL/P patients and with a *P*-value of $\leq$ 0.01. The best results, with $P \leq 0.01$, for common (frequency > 5%) 2- to 4-SNP haplotypes in other candidate loci are presented in Table 8.

Table 8. Most significant results from haplotype-phenotype association analysis of studied genes

					Frequency		
Haplotype	SNP	SNP	SNP	SNP	Cases	Controls	*P*
BCL3							
	rs17728272	rs4803750	rs8100239	rs8103315			
H3	C	A	T	A	0.188	0.109	0.0071
H3	C	*	T	A	0.188	0.109	0.0071
MSX1							
			rs6446693	rs1907998			
H1			C	A	0.444	0.590	$7X10^{-4}$
H2			T	G	0.431	0.308	0.0026
EDN1							
			rs4714384	rs9471438			
H1			T	T	0.496	0.598	0.0179
H2			C	C	0.388	0.278	0.0062
JAG2							
			rs2056860	rs1022431			
H3			T	A	0.125	0.061	0.0069
PVRL2							
			rs519113	rs2075642			
H2			G	G	0.178	0.270	0.0125
H3			C	A	0.228	0.138	0.0054

The most significant associations with CL/P in the *MTHFR* gene were found for common core (H1) haplotypes such as SNP 1-2-3 CTA ($P = 0.0038$), SNP 2-3 TA ($P = 0.0016$), and SNP 1-3 CA ($P = 0.0045$), which were all associated with a higher risk of CL/P. The second most frequent (H2) haplotypes, such as SNP 1-2-3 TGG ($P = 0.0044$), SNP 1-2 TG ($P = 0.0030$), SNP 3-4 GC ($P = 0.0030$), SNP 1-3 TG ($P = 0.0036$), and SNP 1-4 TC ($P = 0.0029$), were associated with a lower risk of CL/P. The strongest signals of haplotype-phenotype association in other candidate loci were found for *MSX1* H2 TG ($P = 0.0026$), *PVRL2* H3 CA ($P = 0.0054$), *BCL3* H3 CATA ($P = 0.0071$), *EDN1* H2 CC ($P = 0.0062$), and *JAG2* H3 TA ($P = 0.0069$) haplotypes, which were associated with a higher risk of CL/P, whereas the *MSX1* H1 CA haplotype (with the lowest *P*-value of 7×10^{-4}), was associated with a lower risk of CL/P. The *P*-values obtained from haplotype-based association analysis performed with markers in *MTHFR, BCL3, EDN1*, and *JAG2* were lower than analyses using individual SNPs.

3. Genes contributing to the risk of nonsyndromic CP in Estonia

We genotyped 630 tag SNPs in 40 candidate genes related to orofacial clefting in 53 patients with nonsyndromic CP and 205 unrelated controls from the Estonian population. Table 9 presents all markers with allelic association test *P*-values of < 0.05 in the CP case-control sample.

In the case-control analysis of the CP phenotype, 37 polymorphisms in 19 genes displayed nominal evidence of association in our study sample.

The most significant association with CP in the Estonian sample was found for SNP rs11624283 ($P = 0.0016$) in the *JAG2* gene, SNPs rs615098 ($P = 0.0018$) and rs629946 ($P = 0.0027$) in the *MMP3* gene, and rs328149 in the *LOXHD1* gene within 'OFC11' linkage region on Chr 18 ($P = 0.0026$). More SNPs of possible interest, rs1106514 in the *MSX1* and rs33992 in the *FGF1* gene, were identified.

Table 9. Most significant results from single-marker association analysis of the CP group

Gene	Chr	SNP	Location	Alleles[a]	MAF[b]		*P*	OR	95% CI
					Cases	Controls			
SKI	1	rs1809822	2221455	A/C	0.0283	0.1073	0.0117	0.2423	0.074–0.796
		rs2172900	2212983	C/T	0.1792	0.1000	0.0233	1.966	1.087–3.553
COL11A1	1	rs3753844	103121048	A/T	0.0283	0.08537	0.0450	0.3121	0.094–1.035
		rs6679803	103175583	G/A	0.2925	0.2024	0.0465	1.628	1.005–2.639
		rs4908291	103345324	T/A	0.5566	0.4488	0.0474	1.542	1.003–2.370
TGF–α	2	rs12473408	70629892	T/C	0.6038	0.4583	0.0076	1.801	1.165–2.783
		rs6743202	70537959	A/T	0.500	0.3756	0.0208	1.662	1.078–2.564
		rs3771485	70609799	C/G	0.3679	0.2707	0.0495	1.568	0.999–2.461
FN1	2	rs4673990	215914298	A/G	0.3585	0.4659	0.0474	0.6407	0.412–0.997
MSX1	4	rs1106514	4926827	G/C	0.4623	0.3122	0.0037	1.894	1.226–2.927
FGF2	4	rs308441	123993515	C/T	0.09434	0.2171	0.0042	0.3757	0.188–0.751
		rs308434	123991278	C/T	0.04717	0.1341	0.0128	0.3195	0.125–0.82
		rs308379	124002346	T/A	0.5	0.3878	0.0364	1.579	1.027–2.425
		rs17408557	124020067	A/G	0.1698	0.1	0.0441	1.841	1.009–3.358
FGF1	5	rs33992	141937322	G/A	0.08654	0.02451	0.0028	3.771	1.491–9.537
		rs34013	141978564	T/A	0.1132	0.2122	0.0211	0.474	0.249–0.904
MMP3	11	rs615098	102225888	G/T	0.2358	0.1171	0.0018	2.328	1.356–3.995
		rs629946	102226906	G/A	0.1132	0.03902	0.0027	3.144	1.439–6.868
		rs17099622	102205562	A/G	0.125	0.04902	0.0049	2.771	1.329–5.778
MMP13	11	rs687558	102338828	A/G	0.4245	0.3146	0.0329	1.607	1.037–1.490
PVRL1	11	rs7103685	119028391	T/C	0.1792	0.2756	0.0427	0.574	0.334–0.987
COL2A1	12	rs1793949	46657862	G/A	0.5189	0.3854	0.0128	1.720	1.119–2.644
		rs6823	46648679	G/C	0.5943	0.4756	0.0293	1.615	1.047–2.492
		rs12228854	46683187	G/T	0.2075	0.1293	0.0415	1.764	1.017–3.061

Gene	Chr	SNP	Location	Alleles[a]	MAF[b]		*P*	OR	95% CI
					Cases	Controls			
JAG2	14	rs11624283	104662016	A/G	0.3302	0.3024	0.0016	0.216	0.077–0.608
		rs2091918	104673664	T/C	0.3585	0.4951	0.0120	0.5698	0.366–0.886
		rs10134946	104725651	C/T	0.4623	0.3463	0.0276	1.622	1.053–2.501
RARA	17	rs506728	35787106	G/C	0.09434	0.178	0.0365	0.4809	0.239–0.967
WNT3	17	rs916888	42218292	T/C	0.2212	0.1415	0.0464	1.723	1.004–2.957
TIMP2	17	rs8080623	74433873	A/G	0.01887	0.08049	0.0245	0.2197	0.052–0.931
OFC11	18	rs328149	42424469	A/G	0.2075	0.1	0.0026	2.357	1.334–4.166
PVRL2	19	rs6859	50073874	G/A	0.5472	0.4268	0.0265	1.623	1.056–2.493
BMP2	22	rs235742	6727526	A/T	0.283	0.1829	0.0225	1.763	1.079–2.881
		rs7270163	6699316	A/G	0.08491	0.1634	0.0420	0.475	0.229–0.987
TIMP3	22	rs10483165	31517342	T/C	0.1604	0.0878	0.0282	1.984	1.066–3.694
		rs137489	31521373	T/C	0.3868	0.3683	0.0489	1.628	0.999–2.653
		rs2283883	31573656	G/T	0.217	0.1293	0.0231	1.867	1.083–3.218

[a] Major allele (in controls) is listed first

[b] MAF = minor allele frequency

SNP = single nucleotide polymorphism; OR = odds ratio; CI = confidence interval.

4. Genes contributing to the risk of nonsyndromic CL/P in Baltic countries

Five hundred and eighty-seven tag SNPs in 40 candidate loci related to orofacial clefting were genotyped in 300 CL/P patients and 606 unrelated healthy controls from Estonian, Latvian, and Lithuanian populations. The average genotype call rate for these SNPs was 99.1%. Genotype distributions among the study groups were in a Hardy-Weinberg equilibrium. The genomic control inflation factor (λ_{GC}) was 1.008 for the entire dataset.

All markers with allelic association test *P*-values below 0.05 (before correction for multiple testing) in the CL/P case-control sample are presented in Table 10. Thirty-three polymorphisms in 16 genes reached the nominal significance level. The strongest association with CL/P was found for SNP rs34010, which is located in intron 2 of the fibroblast growth factor 1 (*FGF1*) gene, where the T allele was associated with a decreased risk (OR = 0.689; 95% CI = 0.559–0.849; $P = 4.56 \times 10^{-4}$). The *WNT9B* rs4968282 minor allele G and the *FOXE1* rs7860144 minor allele A were both associated with decreased risk of CL/P (OR = 0.688; 95% CI = 0.548–0.865; $P = 0.0013$; and OR = 0.723, 95% CI = 0.589–0.889, $P = 0.0021$; respectively). Association with rs34010 did withstand correction for multiple testing after dividing by the number of independent SNPs, taken to be equal to the number of haploblocks (N = 82) within candidate genes ($P_{corr} = 0.037$) assuming that SNPs in one haploblock are not independent. The SNPs rs1907998 and rs6446693, both located 5' of *MSX1*, were associated with CL/P under the assumption of the dominant model of inheritance ($P = 3.97 \times 10^{-4}$ and 5.84×10^{-4}, respectively). Ten markers reached the 1% significance level, revealing *FOXE1, TIMP2, PVRL2,* and *MMP13* genes as additional loci of interest (Table 10).

Stratification of our case-control sample according to sex showed no evidence for a sex-specific component in the association between CL/P and the 10 markers with the strongest association signals ($P < 0.01$). The genotype distributions in male and female cases were similar for these SNPs ($P > 0.05$).

Table 10. Most significant results from single-marker association analysis of nonsyndromic CL/P in Baltic countries

Gene	Chr	SNP	Location	Alleles[a]	MAF[b]		*P*	OR	95% CI
					Cases	Controls			
IRF6	1	rs630065	208065285	T/C	0.285	0.238	0.0290	1.279	1.025–1.596
		rs17389541	208053795	A/G	0.201	0.246	0.0306	0.768	0.605–0.976
FN1	2	rs1404772	215918544	A/C	0.095	0.064	0.0169	1.542	1.078–2.204
		rs10498038	215964589	C/G	0.040	0.064	0.0385	0.612	0.383–0.978
TGF-α	2	rs7605323	70637335	T/C	0.393	0.345	0.0471	1.230	1.002–1.509
MSX1	4	rs6446693	4905981	C/T	0.469	0.407	0.0135	1.286	1.053–1.570
		rs868257	4903074	C/G	0.322	0.274	0.0354	1.256	1.015–1.555
		rs2034461	4896678	G/A	0.130	0.186	0.0394	0.782	0.619–0.988
FGF2	4	rs11737764	124046230	C/T	0.047	0.077	0.0151	0.587	0.380–0.906
		rs308434	123991278	C/T	0.082	0.119	0.0173	0.661	0.469–0.931
		rs308441	123993515	C/T	0.143	0.185	0.0266	0.736	0.561–0.966
FGF1	5	rs34010	141961149	G/T	0.309	0.393	4.56×10^{-4}	0.689	0.559–0.849
EDN1	6	rs16872612	12416068	G/C	0.272	0.225	0.0272	1.290	1.029–1.618
FGFR1	8	rs7829058	38451252	G/C	0.137	0.098	0.0137	1.457	1.079–1.968
		rs6474354	38422122	C/T	0.293	0.247	0.0354	1.265	1.016–1.575
FOXE1	9	rs7860144	99666705	G/A	0.327	0.402	0.0021	0.723	0.589–0.889
		rs874004	99661939	C/G	0.510	0.437	0.0034	1.341	1.101–1.632
		rs973473	99660551	G/T	0.265	0.329	0.0054	0.735	0.591–0.913
MMP13	11	rs7119194	102312606	C/T	0.023	0.049	0.0095	0.465	0.258–0.840
MMP25	16	rs7188573	3051204	T/C	0.370	0.323	0.0463	1.233	1.003–1.516
WNT9B	17	rs4968282	42313936	A/G	0.223	0.295	0.0013	0.688	0.548–0.865
		rs1105127	42322721	G/C	0.392	0.342	0.0377	1.239	1.012–1.518
TIMP2	17	rs7502916	74413228	A/C	0.488	0.419	0.0050	1.325	1.088–1.613
		rs4789936	74409569	C/T	0.548	0.478	0.0052	1.323	1.087–1.611
		rs6501266	74418948	C/T	0.433	0.498	0.0092	0.770	0.632–0.937
		rs7211674	74410660	A/C	0.482	0.424	0.0209	1.261	1.036–1.535
		rs7212662	74429726	T/G	0.490	0.438	0.0398	1.233	1.010–1.505
PVRL2	19	rs519113	50068124	C/G	0.192	0.253	0.0039	0.702	0.552–0.894
		rs2075642	50069307	G/A	0.205	0.161	0.0206	1.347	1.046–1.733

Gene	Chr	SNP	Location	Alleles[a]	MAF[b]		*P*	OR	95% CI
					Cases	Controls			
BMP2	20	rs6085682	6719211	G/A	0.378	0.430	0.0374	0.808	0.661–0.988
		rs6054516	6719073	C/T	0.179	0.142	0.0383	1.322	1.015–1.721
MMP9	22	rs6094237	44062697	A/T	0.407	0.459	0.0362	0.809	0.664–0.987
		rs17576	44073632	A/G	0.363	0.414	0.0471	0.815	0.666–0.998

[a] Major allele (in controls) is listed first

[b] MAF = minor allele frequency

SNP = single nucleotide polymorphism; OR = odds ratio; CI = confidence interval

Haplotype analysis

LD and haplotype analysis were performed with the whole dataset (CL/P patients and controls, N = 906). Eighty-two haplotype blocks were described in selected regions. Case-control analysis was performed for haplotypes constructed from SNPs within LD blocks, and the sliding-window approach was applied. Two haplotypes in *FGF1* reached $P<0.001$, and several haplotypes in seven genes – *FOXE1, FGF1, PVRL2, WNT9B, LHX8, MMP9,* and *TIMP2* – showed $0.001<P<0.01$.

Table 11. Case-control association analysis of haplotypes in *FGF1, FOXE1* and *TIMP2* genes

Haplo-type	SNP 1	SNP 2	SNP 3	SNP 4	Frequency		*P*
					Cases	Controls	
FGF1							
	rs34002	rs250092	rs34010	rs250103			
H2	T	G	T	A	0.288	0.349	0.00921
H1	T	G	T	*	0.308	0.391	5.42×10^{-4}
H4	T	G	G	*	0.173	0.121	0.00274
H2	*	G	T	A	0.288	0.349	0.00914
H2	*	G	T	*	0.309	0.393	5.01×10^{-4}
H2	*	*	T	A	0.287	0.349	0.00794
F0XE1							
	rs10984009	rs973473	rs874004	rs7860144			
H1	G	G	G	G	0.515	0.442	0.00402
H2	G	T	C	A	0.263	0.332	0.00303
H1	G	G	G	*	0.510	0.437	0.00318
H2	G	T	C	*	0.261	0.330	0.00286
H1	*	G	G	G	0.508	0.438	0.00495
H2	*	T	C	A	0.263	0.328	0.00468
H1	G	G	*	*	0.688	0.605	0.00101
H2	G	T	*	*	0.259	0.330	0.00311
H1	*	G	G	*	0.509	0.438	0.00421
H2	*	T	C	*	0.263	0.329	0.00414
H1	*	*	G	G	0.508	0.437	0.00472
H2	*	*	C	A	0.323	0.402	0.00120
TIMP2							
	rs4789936	rs7211674	rs7502916	rs6501266			
H1	C	A	A	T	0.407	0.478	0.00518
H1	C	A	A	*	0.402	0.480	0.00168
H1	*	A	A	T	0.409	0.474	0.00886
H1	*	*	A	T	0.425	0.496	0.00436
H2	*	*	C	C	0.481	0.415	0.00892

[a] Likelihood ratio test for SNP1-3 sliding-window block haplotype effect: $\chi^2= 16.47$ (3 df); $P = 0.0009$

[b] Likelihood ratio test for SNP1-3 sliding-window block haplotype effect: $\chi^2= 14.55$ (3 df); $P = 0.0022$

[c] Likelihood ratio test for SNP1-3 sliding-window block haplotype effect: $\chi^2= 13.69$ (5 df); $P = 0.018$

Table 11 presents the results from haplotype-based association analysis within LD blocks identified at the *FGF1, FOXE1,* and *TIMP2* loci for sliding windows of 2- to 4-SNP haplotypes with a frequency of >5% in CL/P patients and with $P \leq 0.01$.

Table 12. Case-control association analysis of haplotypes in four candidate genes

Haplotype	SNP 1	SNP 2	SNP 3	Frequency		*P*
				Cases	Controls	
WNT9B						
	rs17603901	rs4968282	rs1105127			
H1	C	A	*	0.776	0.705	0.00134[a]
H2	*	G	G	0.223	0.294	0.00155
MMP9						
	rs13038175	rs6094237	rs17576			
H2	G	A	A	0.451	0.387	0.00969
PVRL2						
	rs519113	rs2075642	*			
H3	G	G	*	0.188	0.251	0.00260
LHX8						
	rs17565565	rs6593568	*			
H1	C	A	*	0.077	0.046	0.00610

[a]Likelihood ratio test for SNP1-2 sliding-window block haplotype effect: $\chi^2 = 11.55$ (2 df); $P = 0.0031$

Table 12 shows the results from haplotype-phenotype association analysis for common haplotypes with $P \leq 0.01$ in the *WNT9B, PVRL2, LHX8,* and *MMP9* genes.

The most significant associations with CL/P were found for the second most frequent haplotype rs250092/rs34010 GT and the most frequent haplotype rs34002/rs250092/rs34010 TGT in the *FGF1* gene ($P = 5.01 \times 10^{-4}$ and 5.42×10^{-4}, respectively). Additionally, several common core haplotypes (H1) in *FOXE1* were associated with a higher risk of CL/P, whereas the most frequent haplotypes in *TIMP2* were associated with a lower risk of CL/P. The lowest *P*-values were revealed for common core haplotypes GG in *FOXE1* ($P = 0.00101$) and CAA in *TIMP2* ($P = 0.00168$), and for the second most frequent (H2) haplotype CA in *FOXE1* ($P = 0.00120$).

The strongest signals of association in other candidate genes were found for *WNT9B* CA ($P = 0.00134$) and *LHX8* CA ($P = 0.00610$) haplotypes, which were associated with an increased risk of CL/P. The *WNT9B* GG ($P = 0.00155$) and *PVRL2* GG ($P = 0.00260$) haplotypes were associated with a decreased risk of CL/P.

5. Genes contributing to the risk of nonsyndromic CP in Baltic countries

We genotyped 591 tag SNPs in 40 candidate genes related to orofacial clefting in 104 patients with nonsyndromic CP and 606 unrelated controls from Estonian, Latvian and Lithuanian populations. The average genotype call rate for these SNPs was 99.25%. Genotype distributions among study groups were consistent with Hardy-Weinberg equilibrium.

Table 13 presents all markers having allelic association test P-values below 0.05 (before correction for multiple testing) in the CP case-control sample. Thirty-five polymorphisms in 17 genes displayed nominal evidence of association with the CP phenotype in our study sample, and 10 out of 35 SNPs had P-values less than 0.01.

The most significant association with CP was found for SNP rs17389541, which is located ~8 kb upstream of the interferon regulatory factor 6 (*IRF6*) gene, where the T allele was associated with higher risk (OR = 1.726; 95% CI = 1.263–2.358; $P = 5.45 \times 10^{-4}$). This SNP was significantly associated with CP under the assumption of the recessive model of inheritance ($P = 9.87 \times 10^{-6}$), and association remained significant after correcting for multiple testing.

The A allele of the SNP rs1793949, located in an intron 44 of the collagen type 2 alpha 1 (*COL2A1*) gene, was associated with higher risk of CP (OR = 1.596; 95% CI = 1.235–2.229; $P = 7.26 \times 10^{-4}$). Other markers of interest included the *FGF2, MSX1, FGFR1, WNT3*, and *TIMP3* loci (Table 13). In the case of the *MSX1, FGFR1, WNT3*, and *TIMP3* markers, a variant allele was associated with a risk effect, whereas the variant allele of the *FGF2* SNP had a protective effect.

There was no evidence of a sex-specific component in the association between CL/P and the nine markers with the best *P*-values. The genotype distributions in male and female cases were similar for these SNPs (Armitage's trend test $P > 0.05$).

Table 13. Most significant results from single-marker association analysis of nonsyndromic CP in Baltic countries

Gene	Chr	SNP	Location	Allele 2[a]	MAF[b]		*P*	OR	95% CI
					Cases	Controls			
IRF6	1	rs17389541	208053795	A/G	0.361	0.246	5.45×10^{-4}	1.726	1.263–2.358
		rs9430018	208063165	G/T	0.514	0.440	0.0454	1.351	1.006–1.814
SKI	1	rs12562937	2219338	C/T	0.087	0.151	0.0143	0.534	0.321–0.889
TGF-α	2	rs6743202	70537959	A/T	0.441	0.368	0.0467	1.356	1.004–1.831
FGF2	4	rs308434	123991278	C/T	0.054	0.119	0.0057	0.421	0.224–0.791
		rs308395	123966392	C/G	0.068	0.128	0.0147	0.499	0.283–0.881
		rs2034461	124003745	G/A	0.130	0.186	0.0493	0.652	0.424–1.001
		rs308379	124002346	T/A	0.471	0.386	0.0202	1.419	1.055–1.907
		rs308441	123993515	C/T	0.125	0.185	0.0360	0.629	0.407–0.973
MSX1	4	rs1106514	4926827	G/C	0.438	0.344	0.0095	1.482	1.100–1.998
FGF1	5	rs11167785	142043913	T/C	0.207	0.285	0.0197	0.655	0.456–0.936
		rs7722035	142036157	G/C	0.403	0.487	0.0262	0.712	0.527–0.961
		rs17208908	142040162	G/A	0.192	0.264	0.0280	0.664	0.459–0.959
COL11A2	6	rs213209	33284936	C/T	0.188	0.268	0.0138	0.630	0.435–0.912
		rs9277928	33236438	G/T	0.132	0.192	0.0451	0.637	0.408–0.993
FGFR1	8	rs7829058	38451252	G/C	0.164	0.098	0.0049	1.798	1.189–2.720
		rs2978083	38408060	C/T	0.019	0.061	0.0140	0.300	0.109–0.829
COL2A1	12	rs1793949	46657862	G/A	0.514	0.390	7.26×10^{-4}	1.659	1.235–2.229
		rs6823	46648679	G/C	0.572	0.469	0.0058	1.517	1.270–2.041
		rs12228854	46683187	G/T	0.212	0.139	0.0067	1.663	1.148–2.409
		rs12368284	46659448	A/G	0.317	0.413	0.0093	0.661	0.483–0.904
		rs10875713	46650730	A/T	0.178	0.119	0.0197	1.596	1.074–2.370
		rs11168359	46702290	G/A	0.082	0.141	0.0203	0.544	0.323–0.916
JAG2	14	rs10134946	104725651	C/T	0.442	0.364	0.0318	1.384	1.028–1.864
TIMP2	17	rs7218237	74383233	G/T	0.058	0.104	0.0391	0.530	0.288–0.978
WNT3	17	rs11653738	42242117	T/C	0.414	0.317	0.0064	1.518	1.123–2.053
OFC11	18	rs328149	42424469	A/G	0.178	0.120	0.0213	1.586	1.068–2.356

Gene	Chr	SNP	Location	Allele 2[a]	MAF[b]		*P*	OR	95% CI
					Cases	Controls			
CLPTM1	19	rs5127	50144534	G/T	0.308	0.229	0.0146	1.494	1.081–2.064
		rs16979595	50169221	G/A	0.264	0.198	0.0288	1.457	1.038–2.046
PVRL2	19	rs6859	50073874	G/A	0.476	0.402	0.0472	1.350	1.003–1.816
BMP2	20	rs7270163	6699316	A/G	0.087	0.153	0.0114	0.524	0.315–0.871
		rs235742	6727526	A/T	0.245	0.181	0.0287	1.473	1.039–2.088
		rs1980499	6694498	T/C	0.539	0.459	0.0347	1.375	1.022–1.850
		rs17731603	6720066	A/G	0.135	0.089	0.0375	1.598	1.024–2.493
TIMP3	22	rs242082	31554439	C/T	0.413	0.316	0.0068	1.518	1.121–2.055

[a] Major allele (in controls) is listed first

[b] MAF = minor allele frequency

SNP = single nucleotide polymorphism; OR = odds ratio; CI = confidence interval

Haplotype analysis

LD and haplotype analysis were performed with the whole dataset (CP cases and controls, N = 710). Case-control analysis was performed for haplotypes constructed from SNPs within LD blocks identified in the *IRF6, COL2A1, COL11A2*, *MSX1, CLPTM1, BMP2, WNT3, MMP2, FGF1, FGF2*, *FGFR1, EDN1*, *JAG2, PVRL2*, *SKI, TIMP2,* and *TIMP3* genes. The sliding-window approach was applied.

Table 14 presents the results from haplotype-based association analysis within LD blocks identified at the *COL11A2* and *COL2A1* loci for multiple 2- to 5-SNP haplotypes with a frequency of >5% in nonsyndromic CP patients and with $P \leq 0.01$. The results from haplotype-phenotype association analysis for common haplotypes with $P \leq 0.01$ in the *CLPTM1, BMP2, WNT3, MMP2, FGFR1,* and *MSX1* genes are presented in Table 15.

The most significant association with nonsyndromic CP was found for the second most frequent haplotype rs17389541/rs9430018 GT in the *IRF6* gene (with frequencies of 0.353 in nonsyndromic CP cases and 0.233 in controls, respectively; $P = 2.23 \times 10^{-4}$). Additionally, multiple haplotypes in the *COL11A2* and *COL2A1* genes were associated with a higher risk of non-syndromic CP. The lowest *P*-values in individual LD blocks were revealed for common core haplotypes CAA in *COL2A1* block 2 ($P = 5.76 \times 10^{-4}$) and GC in *COL11A2* block 2 ($P = 9.85 \times 10^{-4}$), and for the second most frequent (H2) haplotype GC in *COL2A1* block 3 ($P = 3.68 \times 10^{-4}$). The most frequent (H1) haplotypes in *COL2A1* block 3, such as GG ($P = 0.0047$) and GGA ($P = 0.0058$), and haplotype CG in *COL2A1* block 2 ($P = 0.0062$) were associated with a lower risk of nonsyndromic CP.

The strongest signals of haplotype-phenotype association in other candidate loci were found for *WNT3* H2 TGA ($P = 0.0035$) and *CLPTM1* H2 TTC ($P = 0.0045$) haplotypes, which were associated with a higher risk of CP, whereas the *FGFR1* H3 CGG haplotype ($P = 0.0040$) was associated with a lower risk of CP. The *P*-values obtained from haplotype-based association analyses performed with *IRF6*, *COL11A2*, *COL2A1*, *CLPTM1*, *WNT3, BMP2*, and *MMP2* markers were lower than analyses using individual SNPs.

Table 14. Haplotype-phenotype association analysis in collagen family genes

Haplotype	SNP 1	SNP 2	SNP 3	SNP 4	SNP 5	Frequency		*P*
						Cases	Controls	
COL11A2 – Block 1								
	rs12526336	rs1547387	rs213209	rs213208	rs2854028			
H1	G	C	C	G	C	0.445	0.342	0.0043
H1	*	C	C	G	C	0.581	0.472	0.0038
H1	G	C	C	*	*	0.577	0.473	0.0057
H1	*	C	C	G	*	0.709	0.603	0.0038
H1	*	*	C	G	C	0.593	0.488	0.0053
H4	*	C	C	*	*	0.712	0.603	0.0029
COL11A2 – Block 2								
	rs9277928	rs3130165	*	*	*			
H1	G	C				0.606	0.477	9.85×10^{-4}
COL2A1 – Block 1								
	rs1635527	rs12228854	rs1859443	rs6580647	*			
H4	G	T	G	C		0.212	0.138	0.0058
H4	G	T	G	*		0.211	0.136	0.0045
H4	*	T	G	C		0.212	0.139	0.0071
H4	G	T	*	*		0.214	0.138	0.0047
H4	*	T	G	*		0.212	0.139	0.0067
COL2A1 – Block 2								
	rs12721428	rs1793949	rs12368284	*	*			
H1	C	A	A			0.514	0.387	5.76×10^{-4}
H2	C	G	G			0.317	0.413	0.0097
H1	C	G	*			0.431	0.534	0.0062
H2	C	A	*			0.513	0.388	6.72×10^{-4}
H1	*	A	A			0.514	0.389	6.78×10^{-4}
H2	*	G	G			0.317	0.412	0.0098

Haplotype	SNP 1	SNP 2	SNP 3	SNP 4	SNP 5	Frequency		*P*
						Cases	Controls	
COL2A1 – Block 3								
	rs12822608	rs6823	rs10875713	*	*			
H1	G	G	A			0.424	0.528	0.0058
H3	G	C	T			0.177	0.112	0.0090
H1	G	G	*			0.425	0.531	0.0047
H2	G	C	*			0.517	0.386	3.68×10^{-4}
H1	*	G	A			0.426	0.527	0.0073

Table 15. Haplotype-phenotype association analysis in six candidate genes

Haplotype	SNP 1	SNP 2	SNP 3	Frequency		*P*
				Cases	Controls	
CLPTM1						
	rs5127	rs10413089	rs3760629			
H2	T	T	C	0.301	0.212	0.0045
WNT3						
	rs199497	rs199496	rs11658976			
H2	T	G	A	0.443	0.339	0.0035
	rs11653738	rs3933652	rs3933653			
H2	C	C	C	0.407	0.314	0.0087
BMP2						
	rs7270163	rs1005464	rs235770			
H4	G	G	C	0.084	0.152	0.0094
MMP2						
	rs837533	rs837535	rs12924764			
H2	A	A	A	0.318	0.232	0.0072
TIMP3						
H2	T	A	G	0.413	0.317	0.0065
H2	T	A	*	0.412	0.317	0.0077
FGFR1						
	rs7012413	rs6996321	rs7829058			
H3	C	G	G	0.156	0.249	0.0040
H1	*C*	*	*G*	*0.517*	*0.616*	*0.0073*
H3	*C*	*	*C*	*0.157*	*0.095*	*0.0072*
MSX1						
	rs1106514	rs12501827	rs12498543			
H1	G	C	*	0.563	0.656	0.0092

DISCUSSION

1. Surveillance system

In Estonia, patients with clefts are treated at the Tartu University Hospital and in the North Estonian Medical Centre. There is no exclusive database for Estonian patients with clefts and therefore it is not possible to include all of the cleft cases in the study. Registration and classification of congenital anomalies in general, and common OCs in particular, is of paramount importance in providing a solid basis for epidemiologic, clinical, and/or fundamental research.

2. Prevalence

For Estonia, we can use only the prevalence findings by Lõvi-Kalnin (1996), conducted between 1970 and 1980. On the basis of the Lõvi-Kalnin study (1996), the rate of occurrence of clefts in Estonia would be 1 case per 777 live births. Therefore, the only way to estimate the number of children affected by clefts is to use the pre-existing information from previous visits to maxillofacial surgeons. Today the occurrence rate of OC is under observation and we cannot report the prevalence of OC in Estonia.

Different ethnic groups have different occurrence rates of different cleft types. Geographical trends for CL/P and other congenital anomalies have been reported in many different studies (EUROCAT http:// www.eurocat.ulster.ac.uk/ pubdata/Publications.html and IBCDMS http://www.icbd.org/). The ethnic differences in facial shape are well recognized (Farkas et al., 2005). It has been suggested that liability to cleft might be related to facial shape (Pashayan and Fraser, 1971; Fraser, 1976) on the basis that parents of children with CL/P have greater bizygomatic width, underdeveloped maxilla and a thinner upper lip than the general population. Unfortunately, good studies comparing cephalometric measures among the European populations are lacking. Facial shape seems to reflect climate (Harvati and Weaver, 2006). Weninger (1979) found, in two quite different geographical regions (Mozambique and Hierro, Canaries), that the maximum head breadth was larger in localities at a higher altitude or/and with a colder climate, than in localities with milder climates.

The differences in cleft prevalence may be related to sun exposure, and studies based on latitude (Grant, 2008; Holick, 2008; Grant and Mohr, 2009) can explain the role of solar UVB and dietary factors in the etiology of malformations (Calzolari et al., 2007). The study by Engström (1982) shows that craniofacial morphology in the rat is influenced by low calcium and low vitamin D. Calzolari et al. (2007) found a positive correlation between isolated CL/P prevalence and latitude, from south to north in Europe.

3. Cleft proportion

In the present study, CL formed 19% of all clefts, CLP formed 42%, and CP 39%. Fogh-Andersen (1942) was the first to emphasize the proportions of occurrence of different cleft types in the Caucasian race: CL: CLP: CP – 1:2:1. The prevalence of CP varies significantly in Europe, not only between registries but also within countries (Calzolari et al., 2007). A recent study from The Netherlands by Luijsterburg and Vermeij-Keers (2011) reported the following cleft proportions: CL (28%): CLP (39%): CP (33%). One of the findings of the present study was a high occurrence rate of CP: CL (19%): CLP (42%): CP (39%) – 1:2:2, which is similar to the studies conducted in Finland and Sweden (Rintala, 1986; Hagberg et al., 1998). The reasons for this finding need further research. Different ethnic groups have different occurrence proportions of different cleft types, and the proportion of isolated CP in general is significantly smaller than the total number of clefts (Natsume and Kawai, 1986).

Across all unilateral CLP and CL, the left side of the face is involved in 2/3 of cases (Wyszynski et al., 1996; Fraser, 1970). According to the present study, the left side of the face was affected 2.2 times more frequently than the right side. No definite explanation for the difference in left- and right-side occurrences is given in the literature. Johnston and Brown (1980) have suggested that blood vessels supplying the right side of the fetal head leave the aortic arch closer to the heart and may be better perfused by blood than those on the left side.

4. Gender ratio

Among Caucasians, men have CLP twice as frequently as women, while CP is more common among women than men (Wyszynski et al., 1996; Calzolari et al., 2007). It also became evident in the present study that significantly more boys were born with CLP (the ratio with girls is 2:1), and girls had CP 1.5 times more frequently than boys. Boys also have more severe diagnoses – there are more CLP patients than CL patients and there are also more bilateral than unilateral cases (Fraser, 1970). According to this study, boys have CLP 2.2 times more frequently than CL, but there are fewer bilateral cases than left-side cases. There is no definite scientific explanation for the differences in clefts, between sexes. One reason given is that the development of clefts occurs at different stages of development in male and female fetuses in the critical stage (Burdi and Silvery, 1969), but there is no justification for this claim.

5. Birthweight

Several studies have shown that the birthweight of children with clefts is similar to the birthweight of children without clefts (Conway, 1966), which was also confirmed by the present study. But the majority of studies demonstrated that

children with CLP presented smaller body dimensions when compared with controls (Marques, 2009; Luijsterburg and Vermeij-Keers, 2011).

6. Parents' age

During the last 20 years, maternal and paternal ages have increased. Some malformations are clearly associated with older maternal age, but the effect of paternal older age is less certain. Increased maternal age is a risk factor for both chromosomal (Hook, 1981) and non-chromosomal abnormalities (Hollier et al., 2000).

In the case of Estonian children with clefts, the mother's age exceeded 30 years in 25% of cases and the father's age exceeded 30 years in 33% of cases. Both parents were older than 30 years in 20% of cases. Half of the mothers were between the age of 20 and 30, and 2.6% were older than 40 years.

An association between advanced maternal age and the occurrence of any type of OC has been found in several studies (Saxen, 1974; Womersly and Stone, 1987; Shaw et al., 1991), but not in all studies (Khoury et al., 1983; Baird et al., 1994; Vieira et al., 2002; González et al., 2008). In this study, no maternal age effect could be observed. Only a few population-based studies were conducted according to the recommendations of the International Consortium for Oral Clefts Genetics (Mitchell et al., 2002), distinguishing between CL/P and CP only, and excluding cases with associated anomalies. A study by Bille et al. (2005), using the population-based Danish Facial Cleft Database, found that the influence of maternal and paternal ages on the risk of CL/P increases with the advancing age of the other parent, and that the influence vanishes if the other parent is young. In contrast, the risk of having a child with CP is influenced only by the father's age, not the mother's age (Bille et al., 2005). Ascertaining whether greater parental age is associated with OC is not only of interest for clarifying the etiology of OC, but is also important from a biological and public-health point of view.

7. Specific exposures

In the present data, over a third of mothers of children with clefts (36%) experienced psychological stress during pregnancy. The main stressors described were problems in the family. However, stress is an important factor in the occurrence of clefts (Fraser, 1970). One fifth of mothers did hard physical work during pregnancy (field work, stock raising) or experienced physical trauma (struck by an animal, domestic violence). Forty five percent of mothers had previously undergone at least one medical abortion and 23% had undergone more than one. One third of mothers (34%) had an exposure to some chemical factor: one fifth (22%) had been exposed to teratogenic toxic substances (fertilizers, various chemicals, medications), 15% had hormonal disorders

during pregnancy (toxicosis during the first trimester or diseases such as diabetes or thyreotoxicosis).

There is little information regarding the temporal sequence between exposure and the outcome of the environmental risk factors, and a dose-response relationship cannot be demonstrated. Not enough information is available to draw any conclusions about the role of these exposures and the risk of oral cleft formation. Many risk factors and mechanisms have been described in the literature for OC (Wyszynski and Beaty, 1996).

8. Candidate gene studies

Candidate gene-based association studies have emerged as a useful tool in the investigation of the genetic component of multifactorial diseases, such as CL/P, as a way to focus on certain regions of interest in the human genome.

Some authors have argued that population-based case-control designs in which candidate genes are used are more suitable than a case-parent design in assessing the effects of risk factors – a crucial step in disease prevention and health promotion (Khoury, 1999). However, family-based studies may still be useful if population stratification is present. The case-parent trio design avoids concerns about spurious results due to population stratification within the sample, primarily because the observed case is always compared with ethnically matched 'pseudocontrols' (parents) (Beaty et al., 2002).

Results from genome scans suggest that several regions may contain genes predisposing to the development of nonsyndromic clefts. On the basis of multiple reports on the association between markers and haplotypes in various genes and OC, we analyzed the role of 18 candidate genes in the Estonian sample for a possible association with CL/P, and 26 SNPs in 9 genes showed nominal *P*-values less than 0.05. The most significant associations with CL/P were found for SNPs in *MSX1, MTHFR,* and *PVRL2,* including several common haplotypes in the *MTHFR* and *MSX1* genes. The association with SNP rs6446693 in the *MSX1* gene region remained statistically significant after correcting for multiple testing.

In the Estonian CP sample, we conducted a study to investigate the role of 40 candidate genes in predisposition to nonsyndromic CP. Six hundred and thirty tag SNPs were genotyped in a sample of 53 CP patients and 205 controls. The strongest associations with nonsyndromic CP were found for *JAG2*, *MMP3, FGF1, MSX1* and in the *LOXHD1* gene within the 'OFC11' linkage region on chromosome 18.

Considering the small sample size of our patient group, we must emphasize that our study carries the risk of false-positive findings as a result of the large number of comparisons performed. Conversely, we cannot exclude the possibility that a modest effect of polymorphisms or haplotypes in disease predisposition may become apparent in a larger sample.

In a follow-up study including neighbouring populations, we genotyped 587 tag SNPs in 40 candidate gene regions, to determine their role in the etiology of CL/P. We genotyped 591 tag SNPs in 40 candidate genes related to orofacial clefting in 104 patients with nonsyndromic CP and 606 unrelated controls in a new clefting sample representing three populations from the Baltic region – Estonians, Latvians and Lithuanians. As they share the same geographic origin, the genetic relatedness of Estonians, Latvians and Lithuanians has been recently confirmed using the principal component analysis, according to the pairwise inflation factor λ and pairwise Fst values between samples (Nelis et al., 2009). We found no evidence of systematic bias due to population stratification as indicated by the genomic control inflation factor (λ_{GC}~1) and the quantile-quantile plots which confirmed the high degree of homogeneity between all three population samples, allowing us to summarize the data of three Baltic countries to increase the study power.

Results from this association analysis suggest that several regions may contain genes predisposing to the development of CL/P. Among the 40 candidate genes analyzed for an association with CL/P, 33 SNPs in 16 genes reached the 5% significance level. The most significant associations were found for SNPs in the *FGF1, FOXE1* and *WNT9B* genes. The strongest evidence of association was found for SNP rs34010 in the *FGF1* gene.

Among the 40 candidate genes analyzed for a possible association with nonsyndromic CP, 35 SNPs in 17 genes showed nominal *P*-values less than 0.05. The strongest evidence of association was found for SNPs rs17389541 in the *IRF6* gene and rs1793949 in *COL2A1*. These associations were not significant after Bonferroni correction, but remained significant after correction by the number of genes evaluated.

8.1. *MTHFR*

Methylenetetrahydrofolate reductase (*MTHFR*) is an important enzyme involved in folate metabolism. Many studies have been undertaken to verify the association between two functional polymorphisms in the *MTHFR* gene – 677 C>T (rs1801133, c.665C>T, p. Ala222Val) and 1298 A>C (rs1801131, c.1286A>C, p. Glu429Ala) – and increased risk of CL/P malformation. However, these studies have provided inconsistent results due to differences in the studied populations as a result of their diverse genetic backgrounds and exposure to varying environmental factors. It has been proposed that low periconceptional folate intake increases the risk of CL/P in offspring, and this risk is even more pronounced in mothers with *MTHFR* 677TT or 1298CC genotypes (van Rooij et al*.,* 2003).

In our study, the *MTHFR* SNPs rs1994798, rs1476413, and rs1801131 (1298 A>C; c.1286A>C, p. Glu429Ala), together with multiple risk and protective haplotypes within the same LD block, showed evidence of association with CL/P in the Estonian study sample. It has been suggested that rs1801131 is not

directly connected to the risk of developing CL/P, but this may be due to near complete LD between 1298 A>C (rs1801131, c.1286A>C, p. Glu429Ala) and 677 C>T (rs1801133, c.665C>T, p. Ala222Val) or disequilibrium with another mutation responsible for the malformation (Pezzetti et al., 2004). Confirmation of an association or linkage between 1298 A>C (rs1801131, c.1286A>C, p. Glu429Ala) and CL/P risk, either separately or in combination with 677 C>T (rs1801133, c.665C>T, p. Ala222Val), has not been found (Beaty et al., 2002; van Rooij et al., 2003). An association between 1298 A>C (rs1801131, c.1286A>C, p. Glu429Ala) and CP cases or mothers was not found in a previous study (Jugessur et al., 2003b). Likewise, the 1298 A>C (rs1801131, c.1286A>C, p. Glu429Ala) variant was not found to be a risk factor for CL/P or CP (Mills et al., 2008). In conclusion, it appears likely that 1298 A>C (rs1801131, c.1286A>C, p. Glu429Ala) and the two other described polymorphisms are not independent risk factors for CL/P, but our data does not exclude a possible involvement of the folate pathway in the development of CL/P.

8.2. *MSX1*

MSX1 has been a plausible candidate gene for clefting. The complete sequencing of the *MSX1* gene demonstrated that point mutations in this gene appear to contribute to approximately 2% of all CL/P cases (Jezewski et al*.,* 2003). Association studies of CL/P (Lidral et al*.,* 1998; Beaty et al., 2002; Vieira et al*.,* 2003; Fallin et al*.,* 2003) and CP (Lidral et al*.,* 1998) have supported a role for *MSX1* in nonsyndromic clefting in different populations. In the Estonian sample from our study, the most significant association with CL/P among all screened candidate genes was found for SNP rs6446693 in the *MSX1* region. The same SNP was associated with CL/P in the Baltic sample. In the Estonian and Baltic CP samples, SNP rs1106514 in the *MSX1* gene was associated with nonsyndromic CP. Case-control studies have reported both positive (Lidral et al., 1998; van den Boogaard et al., 2000; Blanco et al., 2001) as well as negative results. Research data have supported interaction between environmental factors and *MSX1*. The risk of CL/P and CP, related to maternal cigarette smoking and alcohol consumption during pregnancy, increases due to the interaction of such exposure and specific allelic variants at the *MSX1* gene (Romitti et al., 1999). Taken together with several other SNPs and haplotypes demonstrating an association with CL/P and CP, it can be concluded that we have successfully replicated previous findings, showing an association between *MSX1* variants and CL/P and CP.

8.3. *OFC3 (locus 19q13)*

Multiple lines of evidence support the role of one or more genes in the OFC3 region (Chromosome19q13) in clefting. *PVRL2*, located on 19q13, has recently been added to the list of candidate genes hypothesized to play a role in the

etiology of CL/P (Lidral and Murray, 2004). Recent data suggest that both rare and common variants in *PVRL1*, closely related to *PVRL2*, make a minor contribution to nonsyndromic CL/P in multiple populations (Avila et al*.,* 2006; Scapoli et al*.,* 2006). An association study involving five populations did not find an association between CL/P and mutations in the *PVRL2* gene. However, a significant association with an allelic variant in *PVR*, a gene homologous to *PVRL2*, was found (Warrington et al*.,* 2006). In the same study, 16 *PVRL2* variants – 5 common and 11 rare – were identified in cleft patients. We found an association between SNPs rs 519113 and rs2075642 in *PVRL2* and CL/P.

BCL3, a proto-oncogene that encodes a transcription factor involved in cell cycle regulation, has been suggested as a candidate gene for CL/P (Gaspar et al., 2002; Park et al*.,* 2009). The *BCL3* gene has been associated with OC in some association studies (Gaspar et al., 2002; Park et al*.,* 2009), but not others (Fujita et al., 2004; Suazo et al., 2005). A possible reason for these conflicting results is that the susceptibility loci may have different contributions in different populations.

We described associations between CL/P and *BCL3* markers and haplotypes in the Estonian sample, including SNP rs8100239, for which excess maternal transmission has been previously reported in CL/P cases, probably reflecting an imprinting effect or a maternal genotype effect (Park et al*.,* 2009).

A study of a multiplex family in which CL/P segregated with a balanced translocation between 2q11.2 and 19q13.3 suggested that the cleft lip and palate transmembrane 1 (*CLPTM1*) gene, localized to this breakpoint, might play a role in clefting (Yoshiura et al., 1998). Our independent sample showed evidence of association, suggesting the involvement of *CLPTM1* in nonsyndromic CP etiology. In addition, further evidence that the 19q13 region contributes to isolated clefting in heterogeneous populations of European descent has been found (Warrington et al., 2006).

Our work provides further evidence that a 19q13 locus contributes to nonsyndromic clefting in heterogeneous populations of European descent. A previous meta-analysis of 13 genome scans, combining datasets from multiple populations in a linkage analysis to detect candidate loci for CL/P, suggested a role for 19q13 in clefting, supporting our current findings (Marazita et al*.,* 2004).

8.4. *EDN1*

Our study is the first to demonstrate an association between endothelin 1 (*EDN1*) gene variants and haplotypes and CL/P. *EDN1* maps to the chromosomal region of the OFC1 locus (chromosome 6p24-p23) and has been suggested as a candidate gene for CL/P on the basis of the results of linkage studies conducted in populations of different ancestries (Moreno et al*.,* 2004).
Mostly borderline significant association signals combined with evidence presented from epistasis analysis suggest that gene-gene interaction(s) could be a possible mechanism for how *EDN1* exerts its effect as a locus contributing to

CL/P. Four SNPs from the Estonian CL/P sample (rs6912834, rs4714384, rs6906760, rs9471438), and rs16872612 from the Baltic sample, showed associations with CL/P.

There is no information how *EDN1* may influence the development of OC. As *EDN1* is involved in neural crest development this may be the pathway that plays some role; or gene-gene interaction(s) could be a possible mechanism for how *EDN1* exerts its effect as a locus contributing to CL/P.

8.5. *JAG2*

The *JAG2* gene encodes a ligand for the Notch family of transmembrane receptors, which are involved in an essential signalling mechanism required for normal palate development (Casey et al*.,* 2006). In recent family-based association studies, evidence for *JAG2* involvement in CL/P was obtained from haplotype analyses using global tests and single haplotype association tests (Vieira et al*.,* 2005; Scapoli et al*.,* 2008). Interestingly, the most significant data in both studies were obtained with haplotypes that include the nonsynonymous polymorphism, rs1057744. Recently, it has been demonstrated that *IRF6* and Jagged2 function in convergent molecular pathways during oral epithelial differentiation and that this integrated signalling is essential for the control of palatal adhesion and fusion competence (Richardson et al*.,* 2009).

In our study, we found evidence of an allelic association between rs1022431 and CL/P, where allele A was associated with a higher risk of CL/P. This was supported by analysis of haplotypes including this polymorphism. Among all candidate genes screened in this study, the most significant association observed within the Estonian CP sample (P = 0.0016) was for rs11624283 in the *JAG2* gene, and the most significant association observed in the Baltic CP sample (P = 0.0318) was for rs10134946. These results indicate that *JAG2* variants may be involved in the etiology of OC in different populations.

8.6. *IRF6*

IRF6 is one of the CL/P candidate genes with the most accordant results across studies (Vieira, 2008). The most common syndromic form of orofacial clefts is Van der Woude syndrome (VWS), an autosomal dominant disorder characterized by the presence of CL/P or CP and/or lower-lip pits. VWS is caused by mutations in the *IRF6* gene, which belongs to a family of transcription factors that share a highly conserved winged-helix DNA-binding domain and a less conserved protein interaction domain (Kondo et al., 2002). *IRF6* is expressed in the medial edge epithelia of the palatal shelves immediately prior to and during fusion (Knight et al., 2006). Interestingly, VWS is an example of an orofacial syndrome in which cases of CP and CL/P can occur in the same pedigree, suggesting that *IRF6* is probably involved in the fusion process that occurs in both primary and secondary palatogenesis. However, *IRF6* mutations are rare in

families with nonsyndromic orofacial clefts (Jehee et al., 2009). Recently, it has been demonstrated that *IRF6* is essential for oral epithelial differentiation and that *IRF6* plays a key role in the control of palatal adhesion and fusion competence (Richardson et al*.,* 2009). In the present study, from the Baltic CP sample, the *IRF6* SNP rs17389541 showed evidence of association, supported by analysis of haplotypes including this polymorphism, which is a novel implication of *IRF6* in nonsyndromic CP susceptibility. HapMap data from the CEU reference sample indicate that rs17389541 is not in strong LD with the common polymorphism *IRF6* rs642961 (r^2 = 0.057) that was significantly associated with nonsyndromic CL/P and particularly with CL in Europeans, but not with CP (Rahimov et al., 2008). It is likely that association between common variants in the *IRF6* locus and the risk of nonsyndromic CP can be identified in other European populations and that the *IRF6* locus represents an important genetic modifier for this multifactorial malformation.

In our Baltic CL/P sample, we could not demonstrate convincing evidence of an association between CL/P and variants in *IRF6.*

8.7. *FGF* and *FGFR*

Several members of the FGF and FGFR families are expressed during craniofacial development and can, rarely, harbour mutations that result in human clefting syndromes. In a study of Kallmann syndrome patients, cleft palate and dental agenesis were exclusively found associated with *FGFR1* loss-of-function mutations (Albuisson et al., 2005). Animal models also support the involvement of FGFs and FGFRs in the pathogenesis of oral clefting; for example, *FGFR1* hypomorphic mice have CP (Trokovic et al., 2003). The FGF signalling pathway is known to play an important role in craniofacial development, and perturbation of the FGF signal is critical to palatogenesis (Nie et al., 2006). A systematic analysis of genes encoding different FGF proteins and their receptors has highlighted the importance for FGF signalling in nonsyndromic CL/P, and it was suggested that impaired FGF signalling may contribute to 3–5% of CL/P (Riley et al., 2007). In addition, it has been suggested that FGF pathway is involved in interactions with environmental risk factors for CL/P (Pauws and Stanier, 2007). Recently, *FGF12* haplotypes were shown to be significantly associated with CL/P in two Scandinavian samples (Jugessur et al., 2009). In the present study, the *FGF1* SNP rs34010 showed evidence of association in the Baltic sample and *FGF1* SNP rs33992 in the Estonian nonsyndromic CP sample, which is a novel implication of *FGF1* in CL/P susceptibility. Our results give additional evidence that disruptions in the FGF signalling pathway contribute to the pathogenesis of CL/P, also suggesting that association between common variants in the *FGF1* locus and the risk of CL/P can be found in other European populations.

We found associations between nonsyndromic CP and SNPs rs7829058 in *FGFR1* in the Baltic sample and rs308434 in the *FGF2* gene in the Baltic and

Estonian samples. SNP rs08441 was associated with nonsyndromic CP in the Estonian sample. Association with *FGFR1* was supported by haplotype analysis.

Recently, borderline significant association between SNPs in several *FGF*/*FGFR* genes (including *FGFR1*) and nonsyndromic CL/P was demonstrated, and it was suggested that impaired FGF signalling may contribute to non-syndromic CL/P (Riley et al., 2007). *FGFR1* encodes a transmembrane receptor, tyrosine kinase, that transduces signals from secreted FGFs, and insufficient *FGFR1*-mediated signalling during embryonic development may have an impact on palatogenesis in humans and/or mice. It can be concluded that disruptions in the FGF signalling pathway might contribute to non-syndromic clefting phenotypes.

8.8. *FOXE1*

The forkhead box E1 gene (*FOXE1*) is a member of a transcription factor family that contains a DNA-binding forkhead domain and regulates diverse developmental processes in eukaryotes. *Foxe1* is expressed in the secondary palate epithelium in humans at embryonic week 11, and the specific expression pattern of *Foxe1* at the point of fusion between the medial nasal and maxillary processes strongly suggests *FOXE1* as an important player in primary palato-genesis (Trueba et al., 2005; Moreno et al., 2009). Twelve coding variants – missense or silent – in *FOXE1* have been described as rare causes of non-syndromic CL/P (Vieira et al., 2005). Recent studies have implicated SNPs and/or haplotypes clustering in or near *FOXE1* as highly significant risk factors of CL/P, and the described association signals indicated the presence of causal mutations in nearby non-coding regions that regulate *FOXE1* expression (Jugessur et al., 2009; Marazita et al., 2009; Moreno et al., 2009). The three associated SNPs from the current study's Baltic CL/P sample – rs7860144, rs874004 and rs973473 – are located within a 70 kb haplotype block including SNPs rs3758249 and rs4460498, which were the most significantly associated with CL/P in multiple populations (Moreno et al., 2009). Our results support a substantial role for *FOXE1* in the development of CL/P and it's likely that the *FOXE1* locus represents an important genetic modifier for this multifactorial malformation.

8.9. Wnt signalling pathway genes

Wnt signalling pathway genes are involved in craniofacial development and upper lip fusion and are therefore plausible candidates for an etiological role in nonsyndromic clefting. Wnt expression is observed in the upper lip and primary and secondary palates and Wnt signalling mediates regional specification in the vertebrate face (Brugmann et al., 2007). In our Baltic CL/P sample, the *WNT9B* SNP rs4968282 showed evidence of association with CL/P. *WNT9B* lies ~32 kb

telomeric from the start codon of *WNT3*, which is required at the earliest stages of human limb formation and for craniofacial morphogenesis. Furthermore, both *Wnt9b* and *Wnt3* are mapped in the *clf1* locus in clefting-susceptible mice (Juriloff et al., 2005). The gene alterations outside the *WNT9B* coding sequence are expected to cause reduced *WNT9B* gene or protein function in CL/P cases, and *WNT9B* has been suggested as a strong candidate gene for CL/P (Juriloff et al., 2006). The SNP rs197915 downstream of *WNT9B* showed association with CL/P in the European American sample subgroup having a positive family history, whereas the strongest association signals in the entire dataset were reported for SNPs in *WNT3A*, *WNT5A*, and *WNT11* (Chiquet et al., 2008). In contrast, association with rs197915 was not replicated in the Brazilian CL/P sample of Caucasian ancestry, whereas significant association with *WNT3* rs142167 was found (Menezes et al., 2010). In our study, the *WNT3* SNP rs11653738 in the Baltic nonsyndromic CP sample and SNP rs916888 in the Estonian nonsyndromic CP sample, showed evidence of association with nonsyndromic CP. *WNT3*, a human homolog of the *Drosophila* wingless gene, encodes a member of the WNT family known to play key roles in embryonic development, and *WNT3* is required at the earliest stages of human limb formation and for craniofacial development. In a recent study, the strongest association signals were found between SNPs and haplotypes in *WNT3A* and nonsyndromic CL/P, as well as evidence of gene-gene interaction between *WNT3* and *WNT3A* (Chiquet et al., 2008). These findings were extended in a Norwegian sample of nonsyndromic CP case-parent trios where a haplotype effect was reported for the *WNT3A* gene (Jugessur et al., 2009).

Our data support an involvement of the WNT signalling pathway in orofacial development, also suggesting that the 17q21 locus, containing *WNT9B* and *WNT3* genes, contributes to CL/P in populations of European descent.

8.10. *TIMPs* and *MMPs*

Matrix metalloproteinases (MMPs) have been shown to be expressed in the developing secondary palate, associated with the extracellular matrix (ECM) breakdown required for palatal fusion (Brown et al., 2002). MMPs are counteracted by the tissue inhibitors of metalloproteinases (TIMPs), which inhibit MMP activity and thereby reduce excessive proteolytic ECM degradation (Verstappen and Von den Hoff, 2006). During embryonic craniofacial development, MMP and TIMP expression is temporally and spatially regulated to control tissue remodelling, and disruption of their balance can lead to occurrence of malformations, such as CL/P (Blavier et al., 2001). In our Baltic sample, associations with CL/P and CP were found for several SNPs and haplotypes in *TIMP2*, supporting initial findings in a Norwegian sample of CL/P case-parent trios where a haplotype effect was reported for the *TIMP2* gene (Jugessur et al., 2009). Our study provides evidence of the implication of *MMP3* in the occurrence of nonsyndromic CP in the Estonian population.

Further independent studies in other populations with a substantially larger number of individuals should be conducted to verify and extend these results. The inactivation of MMPs by *TIMP2* leads to a failure of palatal fusion in mice (Blavier et al., 2001). Therefore, strict regulation of MMP activity is necessary for a complete fusion of the palatal shelves, and an imbalance in favour of *TIMP2* may increase the risk of CP (Verstappen and Von den Hoff, 2006). The link between CL/P risk and variants in MMP and TIMP genes seems to also rely on alterations in ECM homeostasis and functions.

8.11. *COL2A1* and *COL11A2*

Mutations in genes coding for cartilage collagens II and XI (*COL2A1*, *COL11A1* and *COL11A2*) cause syndromes that are often associated with Pierre-Robin sequence, cleft palate or micrognathia, but it seems possible that sequence variations in collagen II and XI genes may cause or predispose to nonsyndromic conditions in some instances (Melkoniemi et al., 2003). A variety of mutations in the *COL2A1* gene have been identified in families with Stickler syndrome type 1 with systemic features involving Pierre-Robin sequence, severe myopia and/or vitreoretinal phenotype, and osteoarthritis in later life. This study presents the first report describing associations between nonsyndromic CP and several common SNPs, with the strongest association signals found for the intronic SNP rs1793949. In addition, our data showed an association between nonsyndromic CP and certain haplotypes in *COL11A2*, supporting recent findings describing the haplotype effect found for the *COL11A2* gene in a Norwegian sample of nonsyndromic CP case-parent trios (Jugessur et al., 2009). Moreover, one of the chromosomal regions for clefting in mice encompasses the H2 locus, which is homologous to the human HLA locus on 6p21, and includes susceptibility for both corticosteroid-induced and vitamin A-enhanced CP (Murray, 1995). However, we cannot exclude the possibility that observed associations for *COL11A2* could be explained by a limited number of unidentified Stickler syndrome type 3 patients in the study group. As these patients do not present ophthalmic abnormalities and the main conclusive features could be Robin sequence, cleft palate and osteoarthritis, this diagnosis could be frequently missed.

8.12. *TBX22*

Mutations in the T-box transcription factor gene *TBX22* are found in familial and sporadic patients with X-linked cleft palate and ankyloglossia (CPX), which is inherited as a semidominant X-linked disorder. The phenotypic variability in CPX ranges from a mild submucous cleft palate to a severe, complete cleft of the secondary palate. In addition to familial CPX cases, mutations in *TBX22* have been identified in approximately 5–8% of all patients with nonsyndromic CP, and *TBX22* has been proposed to contribute significantly to

the prevalence of nonsyndromic CP across different populations (Marçano et al., 2004; Suphapeetiporn et al., 2007). In a recent study, a functional haplotype variant in the *TBX22* promoter was significantly associated with CP and ankyloglossia (CPA), but association was not significant between cleft palate only (CPO) patients and controls (Pauws et al., 2009). Likewise, our results do not support an involvement of common *TBX22* variants in nonsyndromic CP predisposition. This result is not unexpected, given the heterogeneous nature of nonsyndromic CP and its various confounding factors.

Numerous candidate gene studies have failed to identify either major gene involvement or mutations exerting a major influence on the risk of developing nonsyndromic OC. The failure to pinpoint the molecular events that lead to clefting in humans most likely arises from insufficient knowledge of gene networks and the regulation of gene expression during palatal development. Moreover, it is becoming clear that oral clefts are caused by complex interactions between genetic and environmental variables, which may have different impacts in distinct populations.

As reported previously, Estonians share a relatively common genetic background with the HapMap CEU reference population and several other European populations (Mueller et al., 2005; Montpetit et al., 2006). The genetic relatedness of Estonians, Latvians and Lithuanians, sharing the same geographic origin, has been recently confirmed (Nelis et al., 2009).

Recent GWA studies have reported several new susceptibility loci (2q21, 8q24.21, 10q25.3, 13q31, 15q13 and 17q22) for CL/P in populations of European descent (Birnbaum et al., 2009; Mangold et al., 2010). None of the 40 genes that we selected, before the era of GWA studies, for their potential contribution in clefting lies within the abovementioned chromosomal regions, therefore possible new candidate genes were not included in our selection and associated SNPs in given loci were not genotyped in the overall Baltic sample. However, in a recent independent study, the most significant SNP rs987525 at the 8q24.21 locus was replicated in Estonian and Lithuanian CL/P samples and association with 10q25.3 locus (rs7078160) was replicated in an Estonian sample only (Nikopensius et al., 2009; Nikopensius et al., 2010). The SNPs in four other loci from GWA studies were not replicated in our study.

We acknowledge that a study design based on LD between markers and unobserved etiological variant(s) will have limited power to detect multiple rare variants (e.g. copy number variants) that could also be functionally relevant, and further studies are warranted to confirm reported associations. When investigating the association of single genes with the risk of a complex trait likely to be governed by a considerable number of genes, ORs are almost always low to moderate. This reflects that a specific phenotype results of a combination of different genes, contributing only a small effect, and environmental factors, so taking in consideration the epistasis and gene-environment interactions is appropriate to explain the remaining genetic risk to be identified for the nonsyndromic forms of OCs.

CLINICAL IMPORTANCE AND PRIMARY PREVENTION OF OROFACIAL CLEFTS

Orofacial cleft etiology is heterogeneous. Individuals with OC may experience problems with feeding, speaking, hearing and social integration, which can be corrected to varying degrees by surgery, dental treatment, speech therapy and psychosocial intervention. Identification of risk factors for OC is the first step towards primary prevention. Large, collaborative studies are needed to elucidate environmental and genetic risk factors for orofacial clefts and interactions between them. The recent identification of genes that are likely to influence the risk of nonsyndromic OC, has an impact on genetic counselling in the future and clinical management.

The genetic mechanisms underlying lip and palate development may be due to the disruption of important signalling pathways at various levels that are required for the formation of specific anatomical structures. The challenge is now to perform full-scale genome sequencing in order to identify genetic variants which are more likely to increase the susceptibility to OC. However, estimations of the total genetic contributions to the disease indicate that additional genetic factors involved in OC need to be identified, and both the functional effects of associated variants and the molecular mechanisms behind different pathways must still be ascertained. Further research using large, multicentre, collaborative studies is necessary to identify both genetic and environmental risk factors related to nonsyndromic clefts.

CONCLUSIONS

A combination of epidemiological and clinical approaches may enhance our understanding of the cause and pathogenesis of congenital malformations and may be useful for public health, treatment, and preventive strategies.

The main conclusions of our work are as follows:

1. The present study is the first to present an overview of patients with clefts treated in the Department of Oral and Maxillofacial Surgery of the Tartu University Hospital over the course of 90 years (1910–2000). During this period, 583 patients were recorded (251 females and 332 males; a sex ratio of 1:1.32 respectively).
2. Of the 583 patients with OC, 19% had CL, 39% had CP and 42% had CLP. We found a high occurrence rate of CP among all clefts (CL: CLP: CP – 1:2:2). The most common OC in boys was left-side CLP; in girls it was CP. The left side of the face was damaged 2.2 times more frequently than the right side.
3. Over a third of mothers of children with OC had experienced psychological stress during pregnancy, 45% of mothers had previously undergone at least one medical abortion, and 20% of mothers had been exposed to teratogenic toxic substances. The average birthweight of the children with OC was similar to the birthweight of children without clefts. An association between maternal age and the occurrence of OC could not be observed.
4. The results of this study suggest that several genes known to be involved in craniofacial morphogenesis and/or palatogenesis, may contribute to the incidence of OC.
   - 4.1. Our data provide additional confirmation for the role of *MSX1* and *MTHFR* in the etiology of nonsyndromic CL/P in the Estonian sample.
   - 4.2 We found new supportive evidence that the orofacial clefting locus, OFC3 on Chr 19q13, is probably involved in nonsyndromic CL/P across different populations.
   - 4.3. This study provides, for the first time, evidence of the implication of *IRF6*, *COL2A1*, *COL11A2* and *WNT3* in the occurrence of nonsyndromic CP in north-eastern European populations (Estonians, Latvians, Lithuanians).
   - 4.4. We could not demonstrate convincing evidence of an association between CL/P and *IRF6*, the candidate gene most consistently replicated. This result could be explained by the various confounding factors and heterogeneity among the populations.
   - 4.5. The present study is the first to demonstrate an association between CL/P and common SNPs and haplotypes in *FGF1*, and provides new evidence that variation in the *TIMP2* and *WNT9B* genes contributes to nonsyndromic CL/P.
   - 4.6. We have successfully replicated previous findings implicating *FOXE1* as a susceptibility locus for CL/P across different populations.
   - 4.7 The results of this study underline the importance of the FGF and Wnt signalling pathway genes in the etiology of both CL/P and CP.

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SUMMARY IN ESTONIAN

Mittesündroomse huule- ja suulaelõhe geneetilise etioloogia uuring.

Huule- ja/või suulaelõhe on üks sagedasemaid kaasasündinud väärarenguid, esinemissagedusega 1/700 elussünni kohta, sõltuvalt geograafilistest ja etnilistest variatsioonidest. Huule- ja suulalelõhede kliinilisi klassifikatsioone on erinevaid kuid üldiselt jaotatakse lõhed kaheks suureks haigusfenotüübiks: huule ja/või suulalelõhe (CL/P) ja suulaelõhe (CP). Suulõhede tekkes mängivad olulist rolli keskonna- ja geneetilised tegurid ning elustiiliga seotud riskifaktorid. Sündroomsete suulõhedega seostatud geenide uuringud on näidanud nende olulisust ka mittesündroomsete suulõhede etioloogias. Geneetiliste tegurite olulisus mittesündroomse suulõhe tekkes on kinnitust leidnud perekondade ja kaksikute uuringutes. Kandidaatgeenide uurimisel põhinevad assotsiatsiooniuuringute ja aheldusanalüüside tulemused erinevates populatsioonides on varieeruvad ja vähene kokkulangevus on põhjustatud geneetilisest heterogeensusest ja väikesest uuringute võimsusest.

Suulõhega sündinud lapsed vajavad ravi erinevate spetsialistide poolt ja seda erinevates vanuseperioodided sünnist kuni täiskasvanuks saamiseni ning tihti ka kauem. Suulõhe diagnoos ei ole eluohtlik, kuid neil lastel on kahjustatud paljud olulised funktsioonid nagu: imemine, kõne, keskkõrva ventilatsioon, kuulmine, hingamine ning häirunud on ka näo- ja lõualuude süsteemi areng ning kasv. Need probleemid omakorda võivad põhjustada emotsionaalset stressi, psühholoogilisi probleeme ning võivad mõjutada lapse hakkama saamist koolis. Rehabilitatsioon on võimalik hea meeskonnatööga erinevate spetsialistide vahel ning heas koostöös lapse perekonnaga. Pikka aega kestev ravi on ühiskonnale ka kulukas.

Kuna mittesündroomsete suulõhede etioloogia on multifaktoriaalne, siis väga tihti on raske leida tekkepõhjust, ometi ennetustöö ja inimeste teadlikuse tõstmine võib vähendada lõhega sündinud laste arvu. Suulõhede riskifaktorite teadvustamine ja kaardistamine on esimene samm ennetustöös. Genotüübi ja fenotüübi uuringud võivad tuua uusi teadmisi lähiajal, aga selleks on vaja suuri mitmete keskuste koostöös toimuvaid uuringuid, mis leiaksid seoseid nii keskonnategurite kui ka geneetiliste riskifaktorite vahel.

Eestis puudub riiklik statistika suulõhedega laste sündivuse osas. Huule- ja/või suulaelõhede esmased lõikused tehakse kahes Eesti suurimas haiglas: SA Tartu Ülikooli Kliinikumis ja Põhja-Eesti Regionaalhaiglas. Juhul kui suulõhega lapse vanemad otsustavad pöörduda mujale (välisriiki), siis ei ole võimalik juhtu dokumenteerida.

Käesoleva doktoritöö eesmärgiks oli:

- Ülevaate saamine SA Tartu Üliooli Kliinikumis ravitud suulõhedest ajavahemikul 1910–2000 haiguslugudele tuginedes.
- Erinevate lõhetüüpide esinemise suhte määramine soost lähtuvalt.

- Huule- ja suulaelõhede teket põhjustavate epidemioloogiliste tegurite ja nende esinemise seaduspärasuste leidmine.
- Ananlüüsida kraniofatsiaalses morfogeneesis ja/või patogeneesis osalevate kandidaatgeenide võimalikku seost mittesündroomse CL/P ja CP Eesti ja Kirde-Euroopa (Eesti, Läti, Leedu) uuringurühmas.

Valitud geenidega teostati ühenukleotiidse polümorfismi (SNP) *case-control* assotsiatsioonanalüüsid nii Eesti uuringurühmas kui ka Eesti, Läti ja Leedu ühendatud Kirde-Euroopa uuringurühmas eraldi CL/P ja CP fenotüübiga patsientidel.

Uurimistöö olulisemad tulemused võib kokku võtta järgmiselt:

- Üle pika aja on antud uurimustöö esimene, mille käigus püüdsime saada ülevaate 90 aasta jooksul (1910–2000) Tartu Ülikooli Stomatoloogia kliinikus ravil olnud suulõhedega patsientidest. Säilinud oli 583 dispanseerset kaarti (251 naist ja 332 meest).
- Viiesaja kaheksakümmne kolmest patsiendist 19% esines CL, 39% CP ja 42% CLP. Leidsime suure isoleeritud suulaelõhede esinemissageduse (CL : CLP : CP – 1 : 2 : 2), mis on sarnane Soomes ja Rootsis tehtud uuringutega ja mille põhjused vajavad edaspidist uurimist. Poistel esines sagedamini CLP ja tüdrukutel CP. Vasak näopool oli kahjustatud 2.2 korda sagedamini kui parem pool.
- Üle 1/3 emadest kurtsid psühholoogilist stressi raseduse ajal, 45% emadest olid teinud vähemalt ühe meditsiinilise abordi, 1/5 emadest olid kokku puutunud treatogeensete või toksiliste ainetega. Suulõhedega laste sünnikaal ei erinenud tervete laste keskmisest sünnikaalust.
- Uurimustöö tulemused näitavad et paljud kraniofatsiaalse morfogeneesi ja/või palatogeneesiga seostatud geenid, osalevad suulõhede kujunemisel.
  - *MSX1* ja *MTHFR* geenide järjestuse variandid on seotud mittesündroomse CL/P geneetilise eelsoodumusega Eesti populatsioonis.
  - Leidsime tõendeid, et OFC3 lookus, kromosoomil 19q13 on tõenäoliselt seotud mittesündroomse CL/Pga.
  - *IRF6*, *COL2A1*, *COL11A2* and *WNT3* geenide järjestuse variandid on seotud mittesündroomse CP geneetilise eelsoodumusega Kirde-Euroopa pupulatsioonis (Eesti, Läti, Leedu).
  - Antud uuringus ei leidnud kinnitust kirjanduses erinevates populatsioonides kõige rohkem mainitud ja CL/Pga kandidaatgeenina seostatud *IRF6* geenide järjestuse variantide seos CL/Pga Eesti populatsioonis. Antud leidu võib seletada geneetilise heterogeensusega.
  - Antud uuring on esimene, kus me näitasime, et *FGF1* geeni järjestuse variandid on seotud mittesündroomse CL/P geneetilise eelsoodumusega; ning leidsime kinnitust et *TIMP2* ja *WNT9B* geenide järjestuse variandid on seotud CL/P geneetilise eelsoodumusega Kirde-Eesti populatsioonis.

- Antud uuringu käigus leidsime kinnitust, et teiste populatsioonide uuringutest leitud *FOXE1* geenide järjestuse variandid on seotud CL/P geneetilse eelsoodumusega Kirde-Euroopa pupulatsioonis (Eesti, Läti, Leedu).
- *FGF* ja *Wnt* signaalrajad ning nendes osalevad geenid omavad olulist rolli nii CL/P kui ka CP etioloogias.

Mittesündroomse suulõhe etioloogia kätkeb endas palju erinevaid tegureid ning nende tegurite ja tegurite omavahelise koostoime uurimisega tegeletakse maailmas. Uuringute teostamise teeb raskeks huulelõhede multifaktoriaalne taust ja ka erinevate populatsioonide heterogeensus. Uuringud on näidanud erinevate kandidaatgeenide olulisust erinevates populatsioonides. Vajalik on erinevate regioonide detailne geneetiline kaardistamine haigusseoseliste variantide funktsionaalsete effektide kindlakstegemiseks rahvusvahelises koostöös erinevate uurimisrühmadega ja biopankaadega.

ACKNOWLEDGEMENTS

This study was financially supported by the Estonian Science Foundation grant ETF7076 and by the European Union through the European Regional Development Fund, in the frame of Centre of Excellence in Genomics and grant no. 3.2.0304.11–0312, Targeted Financing from Estonian Government No. SF0180142s08.

I wish to express my deepest gratitude to my supervisors, Docent Mare Saag and Professor Andres Metspalu for the opportunity to conduct this research project and for their critical reading of my dissertation.

I would like to acknowledge the reviewers Professor Katrin Õunap and Priit Kasenõmm for their critical reading of my dissertation.

Personally I would like to thank my colleague Tiit Nikopensius for valuable contribution and for his constructive criticism and support.

I am also very grateful to all the co-authors in Tartu University, Riga Stradins University and Vilnius University.

I am very greatful to dr. Marianne Soots and all department staff in Tartu University Hospital and dr. Kert Karu, dr. Siiri Hanstein from North Estonia Medical Centre for their valuable help collecting patient data and blood samples.

I wish to thank Professor Emeritus Maie Lõvi-Kalnin, for guiding me through the first steps in scientific research in my student years.

I thank all my good friends and colleagues from Department of Stomatology and Ortodontiakeskus for their good company and creating a motivating and friendly atmosphere.

Special thanks go to the gene donors in Estonian Genome Center and all patients and their families for their cooperation.

My hearty thanks go to my parents and family for their understanding and encouragement, for being there for me at the right time and right place.

PUBLICATIONS

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Teadustegevus

Peamised uurimisvaldkonnad:

- Kaasasündinud lõhedega patsientide ning nende lähisugulaste kõvasuulae ja hambakaarte morfoloogia. Huule- ja suulaelõhede teket põhjustavad epidemioloogilised tegurid ja nende esinemise seaduspärasused.
- Hambumusanomaaliate esinemine osteogenesis imerfecta haigetel.
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