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MOLECULAR GENETICS OF NON-SYNDROMIC CLEFT PALATE AND VAN DER WOUDE SYNDROME

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ABBREVIATIONS

bp	base pair
CATCH	cardiac-abnormal facies-thymic hypoplasia-cleft palate-hypocalcemia
cM	centiMorgan
CL	cleft lip
CP	cleft palate
CPH	cleft of the hard palate
CPS	cleft of the soft palate
CPO	cleft palate only
CPSM	submucous cleft palate
CL/P	cleft lip with or without cleft palate
del	deletion
dup	duplication
DNA	deoxyribonucleic acid
IBD	identical by descent
IBS	identical by state
IRF6	interferon regulatory factor 6
LD	linkage disequilibrium
LOD	logarithm of odds
Mb	megabase
MZ	monozygous
NPL	non-parametric linkage analysis
PCR	polymerase chain reaction
RS	Robin sequence
SNP	single nucleotide polymorphism
TDT	transmission disequilibrium test
TGFβ3	transforming growth factor beta3
VWS	Van der Woude syndrome

ABSTRACT

Background Cleft palate is one of the most common congenital malformations. The incidence of non-syndromic cleft palate only is ~1/1000 live births in Finland. The etiopathogenesis of clefts has been widely studied but is still poorly understood. It has been estimated that about half of the cases are nonsyndromic. Nonsyndromic cleft palate is considered to be a genetically complex, multifactorial disease. The aim of our research was to study genetic component influencing on non-syndromic CPO and Van der Woude syndrome (VWS), which is one of the most common cleft syndromes. We also wanted to study the role of collagens in Robin sequence (RS), which is a triad of cleft palate, micrognathia and glossoptosis.

Methods Patients and their families were recruited from the Cleft Center, Helsinki University Hospital. In addition, a few patients were from the USA. Genotyping was done using polymorphic microsatellite markers. Linkage and linkage disequilibrium (LD) between non-syndromic CPO and candidate regions/genes 1p34, 2q32, 22q11, MSX1 and TGFβ3 were analysed using 24 multiplex families. A genome-wide scan was performed in nine of the largest families with non-syndromic CPO and in one large VWS family unlinked to the previously reported VWS locus in 1q32-q41. COL2A1, COL11A1 and COL11A2 were sequenced in 24 RS patients, 17 CPO patients and 21 patients with micrognathia.

Results We found a second locus for VWS in 1p34 that has not previously been reported. Candidate regions/genes did not show any evidence of linkage or LD with non-syndromic CPO. In the genome-wide scan, no significant linkage could be detected, but several interesting regions were found. Two disease-associated mutations were found in COL11A1 and COL11A2 in RS patients. Moreover, two putatively disease-associated mutations were found.

Conclusions Candidate regions/genes 2q32, 22q11, MSX1 and TGFβ3 do not play major roles in cleft palate formation in Finnish multiplex families. Failure to detect significant linkage in the genome-wide scan suggests that there might be multiple genes involved in non-syndromic CPO in Finland. Narrowing down the critical region in 1p34 will be essential in studying the second VWS locus. COL11A1 and COL11A2 have some impact on the Robin sequence but further studies are needed.

Key words cleft palate, Van der Woude syndrome, Robin sequence, genome-wide scan, 1p34, 22q11, 2q32, MSX1, TGFβ3, linkage

INTRODUCTION

Cleft palate is one of the most common congenital malformations worldwide. It can appear as a part of a syndrome, with associated malformations or as isolated, non-syndromic cleft palate only (MIM 119540). It has been estimated that about half of the cases are non-syndromic (Murray 2002). The incidence of non-syndromic CPO is approx. 1/1000 live births in Finland and this is one of the highest seen among white people (Lilius 1992). The autosomal dominantly inherited Van der Woude syndrome (VWS) (MIM 119300) is one of the most common cleft syndromes. The incidence of VWS has been estimated to be 1/34 000 livebirths (Rintala et al. 1985). The Robin sequence (RS) (MIM 216800) denotes a triad of cleft palate, micrognathia and glossoptosis. The Robin sequence is the most common recurrence pattern recognised in syndromic cleft palate patients in Finland (Lilius 1992). It can appear in isolation but it is also seen as a part of another syndrome, most commonly the CATCH and Stickler syndromes (Sheffield et al. 1987, Jones 1997, Holder-Espinasse et al. 2001, van den Elzen et al. 2001).

The etiology and pathogenesis of cleft formation have been extensively studied but it is still poorly understood. On the basis of mouse studies, cleft palate seems to be either a growth or a fusion failure of the secondary palate. In humans, some families with non-syndromic CPO show an autosomal dominant model of inheritance but, in most cases, the model of inheritance is not clearly mendelian. It has been widely accepted that the risk of recurrence is ~ 2 % if one child already has CPO, ~6 % if one parent has it and ~15 % if one child and one parent have it (Curtis et al. 1961). For a monozygous twin the risk is 50-60 % (Murray 2002). These facts clearly show that CPO has a strong genetic component. Numerous previous studies have suggested that many extrinsic factors might influence cleft formation. Thus, non-syndromic CPO and RS are considered to be genetically complex, multifactorial diseases (Murray 1995, Wyszynski et al. 1996, Schutte and Murray 1999, Murray 2002).

On the basis of studies with knockout mice, cytogenetic rearrangements in humans with clefts, identified mutations behind cleft syndromes, and genetic studies on cleft lip and cleft lip with or without cleft palate, several candidate genes and candidate chromosomal regions for CPO exist. No convincing linkage to non-syndromic CPO or non-syndromic RS has been established and, thus, no genes causing non-syndromic CPO or non-syndromic RS have been identified. VWS linked to 1q32-q41 has been found to be caused by mutations in IRF6 gene.

Before and during our study, several association, linkage and mutation studies on CPO have been carried out. None of them has been performed with patients from isolated populations like Finland. No genome scans on CPO have been performed. Also, no genetic heterogeneity in VWS has been reported.

The aim of our study was to map a gene responsible for non-syndromic CPO with the help of Finnish multiplex families. In the beginning, the other aim was to narrow down the VWS region in 1q32-q41. We also wanted to study the roles of collagen genes in RS.

REVIEW OF THE LITERATURE

1 CLEFT PALATE

1.1 Embryology

1.1.1 Normal and abnormal development of the palate

The palate is phylogenetically an old structure. The beginning of the secondary palate is seen in the most primitive reptiles. The development of the secondary palate in mammals has been an important step in the evolution because the palate is a necessary aid in the maintenance of breathing, while the mouth is functioning in eating.

Cleft palate is a common congenital malformation due to unknown etiological mechanisms. Normally the mouth is roofed by the hard and soft palate, which separate the oral cavity from the nasal cavities. The hard palate can be divided into the primary and the secondary palate. In humans the primary palate is anterior to and the secondary palate posterior to the foramen incisivum. The primary palate and the upper lip are formed from the medial nasal process by the end of the seventh developmental week (Sariola 2003). At the same time, two palatal shelves are derived from the maxillar processes (Ferguson 1988). These are composed of mesenchymal cells surrounded by undifferentiated epithelial cells and the extracellular matrix. Unsulphated glycosaminoglycans, collagen and other glycoproteins are the main components of the palatal extracellular matrix (Brown et al. 2002).

Cells in mesenchymal maxillary processi are derived from the neural crest. The neural crest is a temporary organ, which is obviously induced already in the gastrulation phase. Mammalian organs like spinal ganglia and part of the autonomic nervous system are derived from the neural crest. The origins of Schwann cells, glial cells and pigment cells are in the neural crest. Neural crest -derived cells in craniofacial regions differentiate into cartilage, bone, muscle, dental papilla ectomesenchyme, dental follicle ectomesenchyme, sensory and motor ganglia and numerous connective tissue components. Migration of neural crest cells through the complex extracellular matrix to the final locations is a sensitive process.

At first the palatal shelves are vertically positioned on both sides of the tongue in the primary oral cavity. Yet unknown mechanisms make the palatal shelves turn to a horisontal position. Intrinsic tissue pressure caused by hydration of hyaluronic acid may have some impact on shelf elevation (Brown et al. 2002). During developmental weeks seven and eight the shelves fuse to each other, to the primary palate and to the nasal septum. The adhesion takes place between opposite medial edge epithelial (MEE) cells. The loss of the epithelial seam is suggested to be caused by apoptosis, by migration of the MEE cells, or by transformation of MEE cells to mesenchyme (Brown et al. 2002). The fusion is completed in the 10th developmental week. Thus, mainly the formations of primary and secondary palates take place in different developmental weeks (Sariola 2003).

Neither mechanisms of palatal closure, nor the failure of the closure, have been totally resolved yet. The fusion failure was evident in at least two animal studies, because the normal elevations of palatal shelves were seen (Satokata et al. 1994, Kaartinen et al. 1995). Also defective shelf growth, failed elevation or post-fusion rupture of the shelves, have been suggested as a possible mechanism (Ferguson 1988). Decreased motility of the mandibula due

to lack of enough space or to muscle diseases has been proposed to cause cleft palate. Microarray techniques have shown changes in the expressions of numerous genes during murine palatogenesis (Brown et al. 2003) The etiology leading to this disrupted palatal development is considered to include multiple genetic and environmental factors (Schutte et al. 1999, Murray 2002, Carinci et al. 2003).

1.1.2 Animal studies

Prenatal exposure to corticosteroids was first reported to cause CP in rodents. These results have frequently been confirmed (Iida et al. 1988, Marazita et al. 1988, Fawcett et al. 1996, Montenegro et al. 1998). Folate-deficient mice showed delay in palate development (Burgoon et al. 2002). Prenatal folate administration did not reduce the incidence of CP in procarpazine-treated dams, but less severe types of clefts were seen (Bienengraber et al 2001). Prenatal exposure to irradiation increased the incidence of CP in mice (Hiranuma et al. 2000).

Either a deficiency or an excess of vitamin A (retinol) during pregnancy has been repeatedly found to cause CP among other malformations in pigs and rats (Tyan et al. 1987). Prenatal exposure to retinoic acid (RA, oxidized form of retinalaldehyde) produces cleft palate and limb defects in mice (Abbott et al. 1989). Retinoic acid receptor α (RXRα) is involved in the formation of cleft palate induced by RA (Nugent et al. 1999). RA exposure was shown to alter the expression of TGFα, TGFβ1, TGFβ2 and TGFβ3 in embryonic palatal shelves (Abbott et al. 1990, Nugent et al. 1998). RA also inhibits Msx1 mRNA expression in palate mesenchymal cells (Nugent et al. 1998). It should be emphasized that different strains of mice show varying susceptibility to cleft palate induced by drugs (Brown et al. 2002).

Mutations in a very distinct type of genes can lead to cleft palate in mice. These genes encode growth factors, receptors, transcription regulators and enzymes for signalling molecule synthesis. In most of these studies the penetrance is incomplete. In The Transgenic/Targeted Mutation Database (http://tbase.jax.org/) nearly 70 mutated mice are reported to exhibit cleft palate among other malformations.

Msx1 (Hox7) is a member of homeobox containing genes which play important roles during the early development of vertebrates. Murine and human HOX7 genes are structurally very close to each other (Hewitt et al. 1991). Cleft palate and tooth anomalies are seen in Msx1-deficient mice (Satokata et al. 1994). However, Msx1 is not highly expressed in palatal tissue (Nugent et al. 1998). On the other hand, the penetrance of CP in Msx-1 knockouts is 100% (Satokata et al. 1994). Other homeobox genes are also involved in palatogenesis. Non-syndromic cleft palate is seen in Lhx8-deficient mice (Zhao et al. 2000). Lhx8 is a member of the LIM homeobox gene family. Pax9- (Peters et al. 1998), Hoxa-2- (Gendron-Maguire et al. 1993), Mhox- (Martin et al. 1995), Dlx1- (Qiu et al. 1997) and Dlx2- (Qiu et al. 1997) deficient mice exhibit cleft palate and other malformations.

Cleft palate, in addition to small mandible, is seen in Egfr (epidermal growth-factor receptor)-knockouts (Miettinen et al.1999). Abnormal lung development and cleft palate is seen in Tgf (transforming growth-factor) β3-deficient mice (Kaartinen et al. 1995, Proetzel et al. 1995). TGFβ3 regulates the expression of chondroitin sulphate proteoglygan on the surface of medial edge epithelial cells, which have an important role in the fusion of palatal shelves (Gato et al. 2002, Tudela et al. 2002). The expression of TGFβ3 mRNA is mainly seen in mesenchymal-originated cells (Debrynck et al. 1988). Like other transforming growth factors, TGFβ3

controls the proliferation and differentiation of multiple cell types. TGFβ3 maps to 14q24 in humans (ten Dijke et al. 1988).

Cleft palate, among numerous other malformations, is seen in mice lacking Jagged-2 (Jiang et al. 1998), beta-3 GABAA receptor subunit (GABRB3) (Ciuliat et al. 1995, Condie et al. 1997, Homanics et al. 1997), and Sek4 and Nuk receptors (Orioli et al. 1996). Titf2-null mutant mice exhibit cleft palate, in addition to a sublingual or completely absent thyroid gland (DeFelice et al. 1998).

Homozygous mutations in Col2A1 and in Col11A1 cause cleft palate among other malformations in mouse (Seegmiller et al. 1968, Brown et al. 1981). Transgenic mice carrying a dominant mutation in Col10A1 develop craniofacial abnormalities (Chung et al. 1997) Mice deleted for 22q11 region have deficits in sensorimotor gating, and in learning and memory (Paylor et al. 2001)

1.1.3 Extrinsic factors in humans

Epidemiological studies have revealed extrinsic factors which seem to increase the risk of CPO. Overall medicine intake during pregnancy increases the risk (Saxen 1975). Especially the intake of benzodiazepins during the 1st trimester was found to be associated with an increased risk for non-syndromic cleft palate (Saxen 1975). Also antipyretic analgesics other than salicylates and opiates during the 1st, but not during the 2nd or 3rd, trimester, seemed to increase the risk (Saxen 1975). In a recent study, nonsteroidal anti-inflammatory drugs used in early pregnancy were found to increase the risk of CL/P but not of CPO (Ericson et al. 2001). Prenatal exposure to isotretinoin (a synthetic form of retinoic acid) has been reported in newborn affected by cleft palate and other malformations (Benke 1984, Lammer et al. 1985). There have been several reports of newborns who were prenatally exposed to corticosteroids and who suffered from CPO (Doig et al. 1956, Harris et al. 1956). The use of corticosteroids during the 1st trimester was shown to increase the risk of CL/P (Rodriguez-Pinilla et al. 1998) but, unfortunately, no epidemiological studies on corticosteroids and CPO can be found.

Paternal age over 30 years and maternal pelvic X-ray examination prior to pregnancy were also found to be associated with increased risk (Saxen 1975). However, these results have not been replicated. Maternal age was not found to associate with the risk of CPO (Vieira et al. 2002). High birth order seemed to correlate with the risk of both syndromic and nonsyndromic oral clefts (Vieira et al. 2002), but conflicting results were found in an Australian study (Edwards et al. 2003).

Maternal smoking appears to increase the risk more if the mother is a carrier of the rarer allele (Taq1 RFLP) in the TGFα locus (Hwang et al. 1995, Shaw et al. 1996, Shaw et al. 1998). Other studies have not been able to confirm these results (Beaty et al. 1997, Christensen et al. 1998). Maternal smoking only was excluded as a causative factor (Shiono et al. 1986, Khoury et al. 1987, Khoury et al. 1989. Werler et al 1990, Lieff et al. 1999, Mitchell et al. 2001), but conflicting results have also been found (Ericson et al. 1979, Romitti et al. 1999, Beaty et al. 2001). Wyszynski et al. concluded in a meta-analysis that maternal smoking during the first trimester is associated with a higher risk of CPO (Wyszynski et al. 1997). Maternal alcohol consumption during the 1st trimester was also found to be a predisposing factor (Khoury et al. 1989, Lorente et al. 2000) although some others studies did not find any association (Werler et al. 1991, Munger et al. 1996, Shaw et al. 1999, Beaty et al. 2001, Mitchell et al. 2001). Cleft

palate is also seen in patients with fetal alcohol syndrome although cleft lip with or without cleft palate (CL/P) is considered to be more common in FAS (Johnson et al. 1996, Munger et al. 1996)

A positive association between maternal epilepsy and clefts of offspring has been widely discussed. Some studies have shown that maternal epilepsy itself increases the risk of CL/P but not of CP (Abrishamchian et al. 1994), but others have failed to confirm this association (Owens et al. 1985, Friis et al. 1986). The prevalence of epilepsy in fathers, siblings and 2nd degree relatives of CPO patients is not increased compared with the general population (Friis 1989, Hecht et al. 1989, Hecht et al. 1990). On the basis of knock-out mice studies, it has been hypothesised that a decrease in GABAergic transmission might have an impact not only on epilepsy but also on cleft palate formation (Brown et al. 2002). Anticonvulsant drug therapy during pregnancy increases the risk of cleft palate, and this causal relationship has been widely accepted. The use of valproate (Clayton-Smith et al. 1995) or phenytoin (Beghi et al. 2001) during pregnancy is associated wit an increased risk for cleft palate (and also for other malformations). Both phenytoin and valproate decrease serum folate concentration (Berg et al. 1988, Wegner et al. 1992) and sparse intake of folic acid during the first trimester has been suggested to increase the risk of cleft palate (Shaw et al. 1995, Czeizel et al. 1999). Conflicting results have also been published (Hayes et al. 1996).

The methylenetetrahydrofolate reductase (MTFHR) catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, which is a cosubstrate for remethylation of homocysteine to methionine. Methylenetetrahydrofolate reductase deficiency leads to homocystinuria (MIM 236250). Significantly higher plasma homocysteine levels were detected in women carrying fetuses affected with neural tube defects (Mills et al. 1996). C677T mutation reduces the enzyme activity and increases the thermolability of the enzyme, leading to elevated plasma homocysteine levels (Frosst el al. 1995).

The thermolabile MTHFR variant was found to be more common in patients with CPO than in controls (Mills et al. 1999). Children with C677T variant in MTHFR seem to have an approx. two-fold risk of CPO (Jugessur et al. 2003). Maternal multivitamin use in early pregnancy does not clearly decrease the risk of CPO (Itikala et al. 2001, Beaty et al. 2001).

Wyszynski et al. reviewed numerous studies on other potential teratogens in non-syndromic oral clefts (Wyszynski and Beaty 1996). Results of separate studies are often conflicting, which can possibly be explained by variations in genetic and environmental backgrounds (Mitchell et al. 2002).

1.2 Classification and epidemiology

1.2.1 Cleft types

Clefts of the secondary palate can be divided into five subtypes. The most severe form is a cleft of both hard and soft palates (CPH complete). In an incomplete cleft palate (CPH incomplete), a part of the hard palate is closed. The soft palate only can be affected (CPS) and uvula bifida (UB) can be considered as a separate entity or as a subtype of CPS. Sometimes the cleft is covered with mucosa (submucous cleft palate, CPSM). In Finland 13.8 % of cleft palates are submucous (Rintala et al. 1982) Submucous cleft palate might be difficult to

diagnose and, according to McWilliams, only 36 % were diagnosed before the age of 4 years (McWilliams 1991).

1.2.2 Epidemiology of cleft palate only

The worldwide overall incidence of clefts is estimated to be 1/700 with wide variability among races and regions (Murray 2002). Low incidences are seen among black people while the highest incidences are seen among American Indians, Japanese and Chinese (Vanderas 1987). Usually, the incidence of CL/P is higher than the incidence of CPO (Vanderas 1987), but the opposite result has been found in few studies (Vanderas 1987, Saxen 1975, Lilius 1992, FitzPatrick et al. 1994, Jakobsen et al. 2003). Usually, the incidence of cleft lip only is lower than that of CL/P or CPO (Vanderas 1987). In Finland, the incidence of CPO is higher than average, whereas the incidence of CL/P [0.73 per 1000 live births (Lilius 1992)] is lower than average. The ratio of CPO / CL/P (%) in Finland is 59/41 (Lilius 1992). The incidence of non-syndromic cleft palate in Finland was previously reported to be 1.01 per 1000 live births (Lilius 1992). During the years 1993-2001, the incidence of non-syndromic CPO in Finland was reported be 10.6 / 10 000, live- and still births included (Ritvanen, unpublished data). High prevalences of CPO, syndromic forms included, are also seen in the Faroe Islands and Greenland, 1.5 and 1.1 per 1000 live births, respectively (Jakobsen et al. 2003). In north-eastern France, the incidence of non-syndromic CPO was reported to be 0.41 per 1000 live births (Stoll 1991). In Italy, the incidence of non-syndromic CPO was found to be 0.34 per 1000 (Milan et al. 1994). A low prevalence (0.24/1000) of CPO, syndromic forms included, was detected in an Israeli-Arab community (Jaber et al. 2002).

The distribution of probands is not even in Finland; high incidences are seen in regions near Oulu and in central Finland (Lilius 1992). The regional differences are more striking when the birthplaces of grandparents of probands are compared; the Oulu region is heavily overrepresented (Lilius 1992), but, unfortunately, in this study the syndromic forms are also included.

In Finland, 40 % of CP patients are male and 60 % are female but these figures also include syndromic forms (Lilius et al. 1992). Female preponderance, including non-syndromic forms, has also been seen in other studies (Bonaiti et al. 1982, FitzPatrick et al 1994, Milan et al.1994, Robert et al. 1996, Shapira et al. 1999).

1.3 Genetics

1.3.1 Model of inheritance and estimation of numbers of loci

Fogh-Andersen noticed, as early as 1942, that the frequency of CL/P in relatives of a proband with CPO (and vice versa) was not greater than the frequency in the general population, but that the frequency of CPO in first-degree relatives of a proband with CPO was higher than the frequency in the general population (Fogh-Andersen 1942). This result has also been confirmed many times although a few studies have reported opposite results (Vanderas et al. 1987). In a Danish registry study, the sibling risk of non-syndromic CPO was 2.89 (confidence limit 2.01-3.13), while the risk in the general population was 0.058, giving a lambda value 49.8 (Christensen et al. 1996). Heritability denotes the degree to which a given trait or disease is controlled by inheritance. Heritability H can be calculated as (*C*MZ–

*C*DZ)/(100-*C*DZ), where *C* means concordance, MZ monozygous twins and DZ dizygous twins. In Finland, the heritability of CPO was estimated to be 49 % (Nordström et al. 1996). The risk of CPO for MZ twins is greater than 50%, on the basis of literature review (Murray 2002). Wyszynski estimated the concordance rate to be 22 % (Wyszynski et al. 1996). It has been estimated that the risk of recurrence is ~ 2 % if one child already has CPO, ~6 % if one parent has it and ~15 % if one child and one parent have it (Curtis et al. 1961).

Different models of inheritance for non-syndromic CPO have been proposed. Fogh-Andersen proposed, in 1942, an autosomal dominant model with greatly reduced penetrance. Shields et al. analysed Danish CPO pedigrees and proposed the existence of two classes of non-syndromic cleft palate: familial autosomal dominantly inherited CPO and non-familial CPO caused by extrinsic factors like maternal age (Shields et al. 1981). Also, according to Carter et al., some families show an autosomal dominant model of inheritance, while the rest of the families have heterogeneous factors causing CPO (Carter et al. 1982). Fitzpatrick and Farrall proposed an oligogenic model with six loci of equal effect. Demenais et al. could not show any difference between monogenic and polygenic inheritance with a high proportion of sporadic cases (Demenais et al. 1984). Clementi et al. found evidence of a major autosomal recessive locus but only when the penetrance was low and the analysis was limited to CPH (cleft of the hard palate) with no single associated anomaly (Clementi et al. 1997). They did not include CPSM (submucous cleft) in their analysis. The decision to select only CPH for the analysis can be based on the observation made by Christensen and Fogh-Andersen that different subtypes do not segregate within pedigrees (Christensen et al. 1994). The same observation was made by Clementi et al., but there were so few relative-pairs in both studies that statistical significance could not be reached. A single, autosomal recessive locus was found to fit best the CPO data from Latin America (Vieira et al. 2003).

Multiplex CPO families with an autosomal dominant and X-linked recessive model of inheritance have been reported (Jenkins et al. 1980, Shields et al. 1981, Carter et al. 1982, Rollnick et al. 1986). However, non-syndromic cleft palate is commonly considered to be a multifactorial disease with a strong genetic background combined wit a variety of possible extrinsic factors (Murray 1995, Wyszynski et al. 1996, Schutte and Murray 1999, Murray 2002).

1.3.2 Previous molecular and chromosomal studies

Several association, linkage and linkage disequilibrium studies have been performed (Table 1). No indisputable linkage has been reported. Two genome scans for cleft lip with or without cleft palate have been published but no convincing linkage was detected (Prescott et al. 1998, 2000, Marazita et al. 2002). No genome scans for CPO have been published.

Brewer et al. reported two patients with CPO, mild facial dysmorphia and mild learning disability. Both the patients had *de novo* cytogenetic rearrangements involving the same region of chromosome 2q32 (Brewer et al. 1999). Cleft palate is frequently seen in patients with del 4q, dup 3q and dup 10q syndromes. It is occasionally seen in patients with trisomy 8, trisomy 13, trisomy 18, del 3p, del 4p, del 5p, del 9p, del 18p and del 18q syndromes (Jones 1997). Deletions in 4p16-14 and in 4q31-35 are highly significantly associated with cleft palate (Brewer et al. 1998). Duplications in bands 3p24-23, 3p26, 3q23-25, 7q22-32, 8q21, 10p15-11, 14q11-21, 16p12-13 and 22q12-13 are significantly associated with cleft palate (Brewer et al. 1999).

Authors	Material	Methods	Gene	Results
Shiang et al. (1993)	52 isolated CPO patients from Iowa, USA	Association	TGFα	**1**.C2 allele/*Taq*I (p=0.003) **2**. 3 allele/K primer (p=0.17)
Stoll et al.(1993)	57 isolated CPO patients from northern Alsace, France	Association	TGFα	**1**. *Bam*HI *Taq*I No association
Hwang et al. (1995)	69 CPO patients from Maryland, USA	Association	TGFα	**1.** C2 allele/*Taq*I (p=0.015)
Shaw et al. (1996)	141 newborns with CPO from California, USA	Association	TGFα	**1.** A2 allele/*Taq*I OR 1.6 (0.94-2.8)
Lidral et al. (1997)	CPO patients from Philippines	Association	TGFα,TGFβ2, TGFβ3,MSX1	**1.** No association
Beaty et al. (2001)	60 CPO patients from Maryland, USA	Association, linkage disequilibrium	TGFα, TGFβ3, MSX1, BCL3	**1**.TGFα, TGFβ3, BCL3: no association or LD **2.** MSX1: association and LD: allele 4 (p=0.004)
Mitchell et al. (2001)	83 CPO patients from Denmark	Association	TGFβ3, MSX1	**1.** MSX1: no association **2.** TGFβ3: CA allele 2 (p=0.04, p=0.01)
Jugessur et al. (2003)	88 CPO patients from Norway	Association	TGFα, TGFβ3, MSX1	**1.** TGFα: A2/A2 *Taq*I OR 3.2 (1.1-9.2) **2.** MSX1:A4/A4 + TGFα: A2/x OR 9.7 (2.9-32)
Jugessur et al. (2003)	63 CPO patients from Norway	Association	MTHFR	**1.** C667T: OR 2.4 (1.2-4.6) with one allele
Mitchell et al. (2003)	80 newborns with CPO from Denmark	Association	RARA	**1.** No association
Vieira et al. (2003)	24 CPO patients from Latin America	Association	MSX1, TGFβ3	**1.**MSX1: No association **2.** TGFβ3: p=0.02
Hecht et al. (2002)	12 multiplex CPO families, origin not told	Linkage	MSX1	**1.** LOD 2.1 (p=0.01)
Beaty et. al (2002)	83 isolated CPO patients from Maryland, USA	TDT	TGFβ3, MSX1	**1.** D14S61 p<0.005, MSX1 p<0.005
Lidral et al. (1998)	69 CPO patients from Iowa, USA	Linkage disequilibrium, sequencing	TGFα, TGFβ3, MSX1, BCL3, DLX2	**1.**TGFα, BCL3, DLX2: No LD **2.** TGFβ3, MSX1: No mutations, LD between CPO

				and MSX1 CA allele 4
Machida et al. (1999)	89 CPO patients from Iowa, USA	Sequencing	TGFα	**1.** No mutations
Barrow et al. (2002)	2 CPO patients from Iowa, USA	Sequencing	p63	**1.** No mutations
Jezewski et al. 2003	118 CPO patients from Iowa (USA) Japan, Denmark, South America, Philippines, Vietnam	Sequencing	MSX1	**1.** 3/118: 272G>A 451+887G>T 451+1046C>T
FitzPatrick et al. 2003	23 RS patients and 57 CPO patients	Sequencing	SATB2	**1.** No mutations

Table1. Previously reported molecular studies on non-syndromic cleft palate.

1.3.3 Associated syndromes

Cleft palate can be a sign of a syndrome. In Finland, a recognisable syndrome was found in a retrospective study in14.2 % of patients with cleft palate (Lilius 1992). Shprintzen et al. found a higher prevalence for syndromes in cleft palate patients (Shprintzen et al. 1985). It has been suggested that approx. 50% of CP cases are non-syndromic (Jones 1988, Murray 2002). Over 300 syndromes are found with the key word "cleft palate" in Online Mendelian Inheritance in Man (OMIM) database (http://www.ncbi.nlm.nih.gov/Omim/). The three most common syndromes in Finland, according to Lilius, were Robin sequence (3.1 % of all CP patients), van der Woude syndrome (2.3 %) and diastrophic dysplasia (0.6 %). Altogether 39 different syndromes were detected. Twenty-five patients had cleft lip with or without cleft palate but 108 patients had cleft palate only.

Cleft palate associates with some anomalies without any known syndrome. In Finland, 23.1 % of 938 patients with CP (including recognised syndromes) had associated anomalies (Lilius 1992). Cardiovascular anomalies were most common (16 % of all anomalies) and they were strikingly often associated with the submucous type of cleft palate (52 % of all cardiac anomalies in CP patients were detected in patients with CPSM). Anomalies of the lower extremities were the second most common (15.7 % of all anomalies), with club foot being the most common. Anomalies of the central nervous system were the third most common (14.0 % of all anomalies). In northeastern France, 46.7% of patients with cleft palate had associated anomalies (Stoll et al. 2000).

Mutations have been found out in many cleft syndromes. A heterozygous stop mutation in the homeodomain of MSX1 causes Witkop syndrome (MIM 189500), which is a rare syndrome affecting also teeth and nails (Witkop 1965, Jumlongras et al. 2001). A point mutation in MSX1 was found to be cosegregating with dominantly inherited tooth agenesis in a large family (Vastardis et al. 1996). Hemizygous deletions of MSX1 have been demonstrated in some patients with Wolf-Hirschhorn syndrome (MIM 194190), which is considered a

contiguous gene syndrome due to a deletion or a microdeletion in 4p16 (Campbell et al. 1989, Zollino et al. 2003). MSX1 maps to 4p16.1 (Campbell et al. 1989, Padanilam et al. 1992)

Mutations in fibroblast growth-factor receptor 2 (FGFR2) cause Apert syndrome (MIM 101200) (Wilkie et al.1995). Treacher Collins syndrome (MIM 154500) is caused by mutations in TCOF1, which encodes treacle (Treacher Collins Syndrome Collaborative Group 1996). Treacle is a nucleolar protein, but the pathway from mutations to disease has not yet been characterised (Isaac et al. 2000). A sulfate transporter encoding DTDST maps ~900 kb proximal to TCOF1 and is involved in diastrophic dysplasia (Hästbacka et al.1994). Mutations in the T-box transcription factor gene (TBX22) were found to cause X-linked cleft palate (CPX and ankyloglossia) (MIM 303400) (Baybrook et al. 2001). EEC (ectrodactyly-ectodermal dysplasia-cleft lip/ palate, MIM 604292) syndrome is caused by mutations in the *p63* gene, which is a homologue of the tumour suppressor gene *p53* (Celli et al. 1999). Mutations in thyroid transcription factor (TTF-2) cause cleft palate, thyroid dysgenesis and choana atresia (Clifton-Bligh et al. 1998, Castanet et al. 2002). Lymphoedema-distichiasis syndrome (MIM 153400) is caused by mutations a forkhead transcription factor gene (FOXC2) (Fang et al. 2000).

Autosomal recessive ectodermal dysplasia type 4 (MIM 225060) is characterized by CL/P, hypotrichosis and syndactylies. It is caused by mutations in PVRL1 (poliovirus receptor-like 1) (Suzuki et al. 2000). Interestingly, it was found that heterozygosity of one these mutations strongly associates with nonsyndromic CL/P in Venezuela (Sözen et al. 2001). This finding should encourage researchers to study the role of syndromic disorders causing mutations in more common nonsyndromic forms (in any complex disease) (Murray 2001).

Van der Woude syndrome

Van der Woude syndrome (VWS) (MIM 119300) is a dominantly inherited developmental disorder, which was first described by Anne Van der Woude in 1954. The hallmarks of this rare syndrome are pits and/or sinuses of the lower lip, cleft lip and/or cleft palate. The penetrance is estimated to be ~90% (Burdick et al. 1985; Murray et al. 1990; Onofre et al. 1997), and both sexes are equally affected (Burdick et al.1985). Lip pits are present in ~80%, clefts in ~50%, and hypodontia in ~25% of gene carriers (Van der Woude 1954; Rintala et al. 1981, Burdick et al. 1986, Schinzel et al.1986, Kläusler et al. 1987). The incidence of the syndrome is estimated to be 1/34 000 live births (Rintala et al. 1985). VWS was found in ~2% of Finnish cleft patients (Rintala et al. 1985, Lilius 1992). The mutation rate is estimated to be ~1.8 x 10-5 (Burdick et al. 1985).

In 1987, Bocian et al. reported a patient with lip pits and a deletion in 1q32-41. Murray et al. found a linkage between VWS and chromosome 1 q in 1990. In 1995, the region was narrowed down to an interval of 4.1 cM on 1q32-41 (Sander et al. 1995), and further to 1.6 cM in 1996 (Schutte et al. 1996). Microdeletions in 1q32-41 have also been reported in families with VWS (Sander et al. 1994, Schutte et al. 1999). A possible modifying locus at 17p11.2-11.1 was reported when a large Brazilian family was analysed (Sertie et al. 1999). An allele in this locus would enhance the probability of CP in an individual also carrying a defect in the VWS locus. Popliteal pterygium syndrome (MIM 119500) was found to be linked to VWS locus (Lees et al. 1999). A nonsense mutation in exon 4 in interferon regulatory factor 6 (IFR6) was found in a twin affected by VWS (Kondo et al. 2002) Subsequently, mutations in IRF6 in 45 unrelated VWS families and distinct mutations in 13 families affected with

popliteal pterygium syndrome were found (Kondo et al. 2002). They also showed that family members shared the same 18-bp deletion in the IRF6 gene regardless of the different phenotype.

Van der Woude syndrome is of special interest because the phenotype so closely resembles non-syndromic forms of both cleft lip and palate (Schutte et al. 1999).

Robin sequence

A combination of micrognathia, glossoptosis (an abnormal backward and downward fall of the base of the tongue) and an associated cleft of the soft palate is commonly recognised as Robin sequence (MIM 216800) (Gorlin 1990). The sequence was first described as early as 1822 but it bears the name of French stomatologist Pierre Robin who published his observations in 1923. Typically, the cleft is U-shaped in Robin patients, while in non-syndromic cleft patients the cleft is usually V-shaped (Larson et al. 1998, Marques et al. 1998). Increased incidence of twins among Robin patients has been noted (Holder-Espinasse et al 2001, Knottnerus et al. 2001).

Robin sequence is the most common recurrence pattern recognised in syndromic cleft palate patients in Finland (Lilius 1992). Robin sequence can appear in isolated form but it is also seen as a part of another syndrome, the most common being CATCH and Stickler syndrome (Jones 1997, Sheffield et al. 1987, Holder-Espinasse et al. 2001, van den Elzen 2001). In a follow-up study of Robin patients, 7 out of 24 were found to have Stickler syndrome (Sheffield et al. 1987). Van den Elzen found that 63.5% could be categorised as isolated RS, and the remaining 36.8% could be considered syndromic. Holder-Espinasse classified 48 % as non-syndromic. Recently, Houdayer et al. described a patient with RS and interstitial deletion in 2q32.3-q33.2, which is the same CPO-associated region reported by Brewer et al. (Brewer et al. 1999, Houdayer et al. 2001).

Stickler syndrome

Stickler syndrome **(**hereditary arthro-ophthalmopathy) is considered to be the most common autosomal dominant connective tissue disease. The major features are premature degenerative arthropathy, severe progressive myopia with occasional retinal detachment, sensorineural hearing deficit and typical facies usually with maxillary hypoplasia (Stickler et al. 1965, Stickler et al. 2001). Facial dysmorphia, flat face, small mandible, cleft palate are present in 84% of patients (Stickler et al. 2001). The phenotype varies between and within families (Liberfarb et al. 2003). So far, mutations in three different collagen genes have been found to cause Stickler syndrome.

Collagen II is found in cartilage. It is composed of three identical α(II) chains. Stickler sdr type 1 (MIM 108300) is caused by mutations resulting in a premature termination codon in the COL2A1 gene (Ahmad et al. 1991, Brown et al. 1992, Ahmad et al. 1993, Ritvaniemi et al. 1993, Annunen et al. 1999). In addition to Stickler syndrome, defects in COL2A1 cause numerous other diseases (Kuivaniemi et al 1997). COL2A1 maps to12q13.1-q13.2 (Francomano et al. 1987).

Collagen XI is composed of three different α chains encoded by COL2A1, COL11A1 and COL11A2 (Eyre et al. 1987, Ala-Kokko et al. 1995, Vuoristo et al. 1995). Collagen XI belongs to the fibrillar class of collagens and it is expressed in cartilage and the inner ear.

Defects in COL11A1 are the cause of Stickler syndrome type 2 (MIM 604841) (Richards et. al 1996, Sirko-Osadsa et al. 1998). This disorder is characterized by progressive myopia beginning in the first decade of life, vitreo-retinal degeneration, retinal detachment, sensorineural hearing loss, cleft palate, midfacial hypoplasia and osteoarthritis. Marshall syndrome is also caused by mutations in COL11A1 (Annunen et al. 1999). COL11A1 maps 1p21 (Richards et al. 1996).

Defects in COL11A2 cause a form of Stickler sdr (type 3, MIM 184840) which is characterized by midfacial hypoplasia, cleft palate, osteoarthritis, and sensorineural hearing loss, but lacks ocular involvement (Sirko-Osadsa et al. 1998). The lack of ocular involvement is due to the replacement of α2(XI) by α2(V) in the vitreous of the eye (Mayne et al. 1993). COL11A2 maps to 6p21.3 (Brunner et al. 1994). Mutations in COL11A2 also cause non-syndromic hearing loss and, in addition to Stickler syndrome, they are also associated with other autosomal dominant and recessive osteochondrodysplasias (Vikkula et al. 1995, McGuirt et al. 1999, Melkoniemi et al. 2000).

CATCH 22

The incidence of hemizygous 22q11 deletion has been estimated to be 1:4000-6000 live births (Wilson et al. 1994, Botto et al. 2003). Velocardiofacial syndrome (MIM 192430) and DiGeorge syndrome (MIM 188400) are overlapping phenotypes commonly found in patients with 22q11 deletion (de la Chapelle et al. 1981, Goldberg et al 1985, Goldberg et al. 1993, Stevens et al. 1990). Nine percent of these CATCH patients manifest cleft palate (Ryan et al. 1997). Reish et al (2003) found cleft palate in nine out of 38 patients. Patients also have other signs such as velopharyngeal insufficiency, hypocalcaemia, thymic hypoplasia, cardiac problems, renal anomalies, abnormal facies, delayed speech and learning difficulties (Ryan et al. 1997, Somer et al. 1997, Digilio et al. 2003). Of the patients with velopharyngeal incompetence but without overt clefting, 12.5% have the 22q11 deletion (Boorman et al. 2001). It has been estimated that 22q11 deletions may be involved in ~5 % of congenital heart diseases (Wilson 1994). Monozygous twins have been described to exhibit different phenotypes (Singh et al. 2002). The deletions were found to be of maternal origin in 72 % of inherited cases (Demczuk et al. 1995). No studies on sizes of deletions in different tissues have been published. The size of the commonly deleted region is ~3 Mb (Carlson et al. 1997). Polymorphic markers in loci D22S944 and D22S941 are most commonly deleted (Morrow et al. 1995). So far the smallest deletion found has been 20 kb (Yamagishi et al. 1999). The deletion of 20 kilobases removed exons 1 to 3 of the UFDL1 gene, and the patient had typical features of 22q11 deletion (Yamagishi et al. 1999). On the other hand, CATCH is considered to be a “contiguous gene syndrome” (Glover 1995). No hemizygosity of 22q11 was detected in patients with isolated cleft palate (Mingarelli et al. 1996). Routine screening of 22q11 deletion in CPO patients is not recommended (Reish et al. 2003, Ruiter et al. 2003).

2 GENE MAPPING

2.1 Gene mapping in complex diseases

2.1.1 Power estimations

Having enough power to detect an existing linkage is an essential question in gene mapping. The SLINK simulation package is widely used when estimating the power of pedigrees to detect traditional parametric linkage (Ott 1989, Weeks et al. 1990). Simulation methods for population data are used when mapping genetically complex diseases. Population size, "bottleneck" size, genetic drift, number of founders and number of generations are variable parameters. POPSIM and EASYPOP are packages for population simulation (Hampe et al. 1998, Balloux 2001). Recently, methods and a software package for simulations of human genetic data in isolated populations were developed (Ollikainen 2002). These population simulation programs may help the researcher to estimate the sample size and the marker map density. Elegant simulations reduce the amount of work and also the financial costs later.

A recent extensive meta-analysis on 101 genome wide screens for complex diseases revealed that a large sample size and genetic homogeneity were the most important factors promoting successful mapping (Altmuller et al. 2001).

2.1.2 Genetic markers

Restriction fragment length polymorphisms (RFLPs) were the first molecular genetic markers that could be widely utilised in linkage analyses (Botstein et al. 1980). The first genome-wide linkage map was mainly based on RFLPs (Donis-Keller et al. 1987). Minisatellites (VNTRs) were the next step (Jeffreys et al. 1985). Minisatellites usually have a length of over 1000 bp, which makes it difficult to use PCR-based methods. Short tandem repeat polymorphisms (STRPs) made analyses more rapid, and also the degree of heterozygosity is higher than in RFLPs or VNTRs (Weber et al. 1989). Microsatellites are di-, tri- or tetranucleotide repeats which have provided the main skeleton of the genome-wide human linkage maps (Gyapay et al. 1994, Murray et al. 1995, Sheffield et al. 1995). The nucleotide repeats must be amplified in polymerase chain reactions (PCR) (Mullis et al. 1986), and the repeat sizes are separated in gels with the help of electrophoresis. The alleles are visualised by silver staining, radioactive or fluorescence labelling.

Single nucleotide polymorphisms (SNPs) are bi-allelic markers, which allow highly automated genotyping (Kruglyak 1997, Collins et al. 1998). They are estimated to exist in approx. every 1000^{th} bp, and their total number is estimated to be ten million (Sachidanandam et al. 2001, Kruglyak and Nickerson 2001). To obtain maximal information, a map must be dense enough because allele information is based only on two possible polymorphisms. In fact, map density was shown to be more critical than marker heterozygosity (Kruglyak 1997).

2.1.3 Linkage analysis

Linkage means cosegregation of a trait and a marker. If they are physically closely tightened to each other, the probability of cross-over is very small. Crossing-over produces a new combination of alleles between trait and marker loci. The longer the distance between these two loci, the more probable is the crossing-over event. The proportion of rearranged

chromosomes after meiosis is called the recombination fraction. The shorter the distance between two loci, the smaller the recombination fraction is. This distance can be represented as Morgans (M). Within a distance of one cM, the recombination fraction is approx. 1 %. The length of the human genome is approx. 3000 cM.

Linkage analysis tries to localize a gene with the help of polymorphic markers. If a particular allele is identical-by-descent in all affected members of the same family, one can suspect linkage. If similar cosegregation of particular alleles in a given maker locus takes place in many families, one can start to count how probable it is that this kind of inheritance pattern can happen by chance. If the probability of chance is 1×10^{-3}, the logarithm of odds (LOD) is 3 (Morton 1955). In the case of single-gene Mendelian disorder, a LOD score below -2 indicates a region where the possibility of linkage can be disregarded (Morton 1955). Usually, the linkage is regarded as established when the LOD score is ≥3. This corresponds to a 5% significance level in two-point analysis, and a 9% significance level in multipoint analysis.

The LOD scores are calculated with the help of computer programs. The first computer program (Liped) for linkage analysis of human pedigrees larger than two-generation was written by Ott (Ott 1974). MLINK (LINKAGE package) was the first program to perform multipoint analysis (Lathrop et al. 1984). FASTLINK (Cottingham et al. 1993) is a newer and improved version of the LINKAGE package. The user of these programs needs to input accurate parameters in disease models. This demand is difficult when dealing with genetically complex, multifactorial diseases. The affected-pedigree-member method (APM) compares IBS (identical-by-state) sharing among affected individuals with IBS sharing expected under random segregation (Weeks et al 1988). Multipoint sib-pair analysis can be performed for example in a computer package, MAPMAKER/SIBS (Kruglyak et al. 1995). To extract more information from a pedigree, Kruglyak et al. applied non-parametric linkage analysis (NPL) into Genehunter package (Kruglyak et al. 1996). The NPL score (Z) announces whether affected individuals share IBD (identical-by-descent) alleles more often than expected by chance.

An association between a disease and a particular allele in a marker locus can be a result of linkage disequilibrium (LD). LD means a non-random association of alleles in linked loci. It depends on the age of mutation and the recombination frequency (Jorde 1995). The LD measure increases when particular alleles of two linked loci cosegregate more often than expected by chance. LD mapping is especially powerful in isolated populations where one or a few founder mutations are expected to have taken place (de la Chapelle 1993, Jorde 1995). The genetic distance between a disease gene and a marker locus can be estimated on the basis of LD and applying the Luria-Delbruck principle (Hästbacka et al. 1992, de la Chapelle 1993). This method was successfully used when mapping the DTD gene (Hästbacka et al. 1992).

Recently, Kruglyak estimated that when mapping common disease genes, the useful level of LD in general and in isolated populations is unlikely to be more than 3 kb and that would require about half a million SNPs for whole-genome studies (Kruglyak 1999). On the other hand, he demonstrated that the extent of LD can be larger in populations where the bottleneck has been very narrow or if the frequency of the rarer marker allele is very low.

The transmission disequilibrium test (TDT) detects linkage between a disease locus and a marker locus in the presence of association (Spielman et al. 1993). The TDT studies a transmission distortion of alleles transmitted to an affected offspring from an affected parent

compared to untransmitted alleles. The statistical significance of the TDT is tested by $\chi 2$ or by the exact binomial test (Spielman et al. 1993). In multiplex families with many generations, the TDT is a valid test for linkage but not for LD. In multigeneration families, a false-positive "LD" can be seen because of non-independent observations.

2.2 Finland - the northern isolation

2.2.1 Short review of the history of the Finnish population

The Finnish population was 5 206 295 at the end of 2002 (Statistics of Finland). The growth has been rapid, since the number was ~2 656 000 in 1900. It has been estimated that the very first immigrants settled in Finland for about 9000 years ago (Virrankoski 2001). The first two bigger waves of settlement seem to have taken place around 3200 and 2000 B.C.. These immigrants might have arrived from the east. Immigration from the west took place around 1200 B.C., and those immigrants settled mainly in western Finland (Koskinen et al. 1994). The size of the population was still extremely small. It has been estimated that during the whole prehistorian time the size of the population has been at most 5 000 - 10 000 inhabitants (Jutikkala 1996). At the end of the prehistorian time, only few regions, mainly in the coastline, were inhabited (Virrankoski 2001). It is important to notice that large regions of Finland were inhabited only a few hundred years ago. During the period of this so called late settlement the relative growth of population was the most rapid.

Researchers have not been able to trace the precise origins of the Finns. According to mitochondrial and genomic DNA diversity, the Finns, with the exception of the Saami, seem to be genetically indistinguishable from many other European populations (Lahermo et al. 1996). On the other hand, analysis of Y chromosomal diversity suggests the possible origin for at least a part of the Finnish population in Northern Eurasia (Lahermo et al. 1999). This finding supports previous theories based on linguistic analysis (Wiik 1997).

Between the years 1698 and 1721, the population diminished greatly because of starvation due to poor harvests. This period is considered to be a one bottleneck of the Finnish gene pool. A relative and very effective bottleneck was the emigration from Savo to northern parts of Finland in the 16th century (Norio 2000). Norio divides Finland roughly into two areas: the region of the old settlement and the region of the young settlement. The traces of bottlenecks were seen when Y chromosomal polymorphisms were analysed: the pattern of haplotype diversity in Finnish males was strikingly narrower than in other European populations analysed (Lahermo et al. 1999). Also the high prevalence of some recessive diseases, that are very rare in other countries, reflects bottlenecks followed by a quite rapid expansion of the surviving gene pool.

2.2.2 The Finnish disease heritage

2.2.2.1 Single-gene disorders

"The Perheentupa stairs" give an informative view of the hereditary diseases which have a higher relative incidence in Finland than anywhere else (Perheentupa 1972, Norio 2000). The majority of the genes and their defects behind these diseases have already been identified and molecular events are being studied at the moment (Peltonen et al. 1999). The enrichment of

these disease genes, combined with well-organised church registers and the high level of medical research facilities, have provided an excellent basis for genetic research. The model of inheritance in these diseases is recessive, with the exceptions of an autosomal dominant model in two and a X-chromosomal recessive model in two diseases. The most common are the recessive disorders aspartylglucosaminuria (AGU) (Palo 1967, Ikonen et al.1991), congenital nephrotic syndrome (CNF) (Norio 1966, Männikkö et al. 1995), and infantile neuronal ceroid lipofuscinosis (INCL) (Hagberg et al. 1968, Vesa et al. 1995). The sizes of regions showing linkage disequilibrium flanking the disease genes are approx. 3 cM, 3 cM and 2,5 cM, respectively. The mutations seem to have taken place about for 80-120 generations ago, i.e. 2000-3000 years ago.

Salla disease (Aula et al. 1979, Verheijen et al. 1999), Northern epilepsy syndrome (EPMR) (Hirvasniemi et al 1991, Ranta et al. 1999) and vLINCL (Santavuori et al 1982, Savukoski et al 1998) represent newer mutations. The birthplaces of grandparents of EPMR patients are located in the Kainuu region and of vLINCL patients in the Ostrobothnia region (Varilo 1999). In vLINCL, LD covering a distance of 11 cM was detected and the mutation was estimated to have taken place about 500 years ago (Varilo et al. 1996). In Salla disease the interval for LD is also approx. 10 cM (Schleutker et al. 1995).

2.2.2.2 Genetically complex diseases

Tracing genes involved in multifactorial diseases has been shown to be a demanding task. Genome-wide scans of several complex diseases in Finnish patients have been performed. Several examples are listed here. Linkage between non-insulin dependent diabetes mellitus and chromosome 12 was established (Mahtani et al. 1996). A genome-wide search was performed in Finnish multiple sclerosis families mainly originating from Ostrobothnia (Kuokkanen et al. 1997). The prevalence of MS is clearly increased in Ostrobothnia (Kinnunen et al. 1983). A suggestive linkage to 17q22-q24 was established. Multiple suggestive loci were found when a genome-wide scan was performed in Finnish schizophrenia patients (Hovatta et al. 1999). No LD was detected although patients originated from a restricted Kuusamo area (Varilo et al. 1999). A genome-wide scan for elevated diastolic blood pressure revealed linkage to the AT_1 gene (Perola et al. 2000). A genome-wide scan of obesity in Finnish sibpairs revealed linkage to Xq24 (Öhman et al. 2000). Evidence for linkage between 7p14-p15 and three phenotypes related to asthma (asthma, a high serum IgE level and a combination of the phenotypes) in a Finnish subpopulation from the Kainuu region was detected in a genome-wide search (Laitinen et al. 2001). Autism-spectrum disorders were found to be linked to 3q25-27 in Finnish families (Auranen et al. 2002). Also a linkage between coeliac disease and 15q11-q13 was found when using a subpopulation from the northeastern part of Finland (Woolley et al. 2002).

AIMS OF THE STUDY

1 To localize a gene responsible for non-syndromic cleft palate in Finnish patients.

2 To narrow down the critical region in the van der Woude syndrome. While we were doing this, we found genetic heterogeneity in Finnish VWS families and that result encouraged us to map a second locus involved in VWS.

3 To find out if mutations in COL2A1, COL11A1 and COL11A2 cause Robin sequence, non-syndromic cleft palate or micrognathia.

SUBJECTS AND METHODS

3.1 Ethical issues

The research plan was approved by the Social and Health Ministry of Finland, by the ethical committee of the University Hospital of Helsinki and by the ethical committee of the Department of Medical Genetics of Helsinki University. The patients were contacted from the University Hospital of Helsinki, where they are or have been treated medically. The relatives were contacted with the permission of the patient. The informed consents were obtained before taking any samples. No individual data will be published. Personal results (i.e. genotypes, haplotypes, carrier status) will not be told to participants. All the data concerning the patient's disease, family history and research results are confidential. These facts were written in the informed consent.

Every participant was given a study-specific ID number. ID-numbered samples without names were handled in the laboratory. The connection between names and numbers is in a database. Access to the database is permitted only for the researchers named in the research plan and approved by the ethical committees.

3.2 Patients

Participants were collected from the records of the Cleft Center. Cleft patients in Finland have been treated centrally since 1948 at the Red Cross Hospital for Plastic Surgery, which then became, in 1984, the Plastic Surgery Unit of the I Department of Surgery, Helsinki University Central Hospital. We searched the medical records of all patients with cleft palate from the years 1967-1996, those years included. All types of cleft palates were accepted. We contacted 250 patients by sending them a letter. The patients were chosen on the basis of three criteria: 1. According to the medical record they did not seem to have other malformations or syndromes, 2. They had reported of at least one similarly affected relative 3. They could still be reached by mail.

Twenty-four multiplex CPO pedigrees were chosen for the DNA analysis of candidate regions (Figs 1-3). The pedigrees consisted of 63 affected, and 112 unaffected, a total of 175 individuals. Families came from different regions of Finland. Probands and as many affected relatives as possible were examined to rule out undiagnosed syndromes. The whole nuclear family was asked to meet the examiner, if possible. Family members were then examined and dysmorphic features were searched for.

Five families with many members affected by VWS were chosen for the VWS study. In the VWS families, 56 individuals were genotyped, of whom 25 were affected (Fig 4). Family 0057 was re-examined in purpose to confirm the diagnosis and to determine the affection status of all pedigree members.

During the years 1967-1996, 103 patients with Robin sequence were treated in the Cleft Center. Ninety-three of them were contacted by sending them a letter. Thirty-three answered and were willing to participate. Ten out of these 33 were excluded on the basis of a confirmed or suspected syndrome. Of the remaining 23 patients, seven had had severe breathing difficulties immediately after delivery. The RS diagnosis was based on cleft palate and small mandibula in the rest of the patients. Seven patients reported a similarly affected relative but

only in one case was the relative a first-degree one. Patients were not re-examined but the information was collected by questionnaries, telephone interviews and from the medical records in the Cleft Center. Altogether 150 controls, whose samples were analysed together with the samples of RS patients, were individuals without any cleft and without relatives with clefts.

One patient with non-syndromic Robin sequence (patient no. 62) was recruited from the USA. Seventeen of the 21 patients with non-syndromic micrognathia were recruited from the Center for Craniofacial Disorders and Department of Dentistry at Montefiore Medical Center, Bronx, New York, and the remaining four from the maxillofacial surgery service at the University Hospitals of Cleveland (patients 41 to 61). The medical records and samples of these 22 patients from abroad were sent directly from USA to Oulu.

3.3 Family history and genealogical studies

Patients were asked about the family names and birth places of their parents and grandparents. They were also asked for information about any similarly affected relatives, i.e. relatives with CL, CL/P or CP. Names of affected relatives were asked for to try to connect the families to each other. Finnish pedigrees were also expanded with the help of the Finnish church records. Local church records usually show pedigree information on about five generations backwards. Ancestors born before ~1860 can be traced with the help of church records in the Finnish National Archives. In our study we used patients' own knowledge about their ancestors and the local church records to build the pedigrees.

3.4 DNA samples

Blood samples were taken either in local health centers or in the University Hospital of Helsinki. EDTA-preserved venous blood was either frozen or DNA was immediately extracted. DNA was extracted non-enzymatically from leukocytes (Lahiri et al. 1991). In this method no organic solvents are used. Five ml of blood was mixed with 5 ml TKM 1 + P40 buffer for cell lysis. TKM 1 consists of 10 mM Tris-HCl pH 7.6, 10 mM KCl, 10 mM $MgCl_2$, and 2 mM EDTA. The solvent was centrifuged at 2200 RPM for 10 min, then the nuclear pellet was washed with 5 ml of TKM 1 buffer and centrifuged again as above. The pellet was then suspended in 800 µl TKM 2 buffer. TKM 2 buffer consists of 10 mM Tris-HCl pH 7.6, 10 mM KCl, 10 mM $MgCl_2$, 0.4 M NaCl and 2 mM EDTA. Fifty µl of 20 % SDS was added and the suspension was mixed by pipetting back and forth. The tubes were incubated 1-2 days at 60° C. Then, 360 µl 5M NaCl was added for protein precipitation. The DNA-containing supernatant was then separated by centrifuging at 12000 RPM for 10 min. DNA was precipitated by adding 3 ml of cold 100% ethanol. Precipitated DNA was mechanically transferred to tubes containing 70% ethanol. Finally, DNA was dried in the air and dissolved in TE buffer to be preserved. DNA was preserved frozen.

3.5 Microsatellite markers

Linkage and association were searched among four candidate regions and non-syndromic CPO among 24 multiplex families. The 22q11 region was studied using nine polymorphic markers which are inside the 3 Mb region commonly deleted in patients with velocardiofacial syndrome (Morrow et al. 1995). Markers at loci D22S941, D22S944, D22S264, D22S311, D22306, D22S308 and D22S425 were ordered from Research Genetics

(http://www.resgen.com). Markers at loci D22S1638 and D22S1623 were developed at Genosys (http://www.sigma-genosys.com). TGFβ3 in chromosome 14 was studied using polymorphic markers at loci D14S273 and D14S61 which were ordered from Research Genetics. TGFβ3 is located between these markers in YAC 746B4, which has a size of 1800 kb (Cruts et al.1995). The MSX1 region in chromosome 4 was studied using a polymorphic marker D4S394, which is located c. 7 cM proximal to MSX1, and with an intragenic dinucleotide (CA) repeat polymorphism. In addition, the entire chromosome 4 was analyzed using 20 polymorphic markers from the modified Weber set VI (http://www.pebio.com). The mean distance between the markers was 11.3 cM. The critical 2q32 region was further analyzed using polymorphic markers D2S311, D2S348, D2S2392 and D2S115 (Brewer et al 1999, Hadano et al. 1999). The entire chromosome 2 was also analyzed using markers from the ABI marker set. The mean interval between the 21 markers was 13.8 cM.

The genome-wide screen was performed in nine largest multiplex families with non-syndromic CP. The genome scan was performed in the Finnish Genome Center (http://www.genome.helsinki.fi). The 377 fluorescent polymorphic markers used were from the Applied Biosystems Linkage mapping Set MD-10, Foster City, USA (http://www.appliedbiosystems.com).

In the genome-wide scan at stage II, six additional markers (D1S247, D1S513, D1S2723, D1S380, D1S1188, D1S2722) (see below) were used to obtain maximal information from the interesting VWS region in 1p34. Thus, 1p34 and the entire chromosomes 2 and 4 were screened in 24 multiplex families. The interval between markers in chromosomes 2 and 4 in the first nine families was ~ 5 cM, while it was ~ 10 cM in the remaining 15 families.

Linkage was tested between VWS and 1q32-q41 and between VWS and a proposed modifying locus in 17p11.2-p11.1. At the first stage, nine polymorphic markers (D1S1663, D1S245, D1S491, D1S3754, D1S2136, D1S3753, D1S205 and D1S414 (Schutte et al. 1999) were genotyped in 1q32-q41. The genetic distances are provided by the Sanger Center (http://www.sanger.ac.uk/HGP/Chr1/). Because of the exclusion of the linkage in family 0057 and uninformative results in family 0062, an additional 25 markers were genotyped in chromosome 1. These markers were from the modified Weber set VI (http://www.pebio.com). In family 0057, the genome-wide screen was performed with 381 polymorphic microsatellite markers from ABI PRISM Linkage Mapping Set-MD10 (Applied Biosystems). For the second stage of analysis of family 0057, six additional markers (D1S247, D1S513, D1S2723, D1S380, D1S1188, D1S2722) within the region flanked by markers D1S234 and D1S2797 were genotyped. The positions of these markers were ascertained from the Marshfield comprehensive human genetic map (http://www.marshmed.org/genetics/).

Microsatellite polymorphisms were amplified by PCR (Mullis 1986). When PCR products were visualised with silver staining, fifty ng of DNA was amplified in 20 µl reaction with 20 µM of each primer, 200 µM of dNTPs, 2.0 µl of buffer, 1 unit of Amplitaq Gold enzyme and 4.68 µl of H_20. The cycling conditions were within the following ranges: 94°C for 10 min, 30 cycles at 94° C for 30 s, 53-58°C for 35 s, 72°C for 30 s, and 72°C for 10 min. When PCR products were visualised with fluorescent dyes, 50 ng of DNA was amplified in 15 µl reaction with 5 µM of each primer, 300 µM of dNTPs, 1.5 µl of buffer, 0.15 µl of Amplitaq Gold enzyme 5 U/µl and 0.35 µl of H_2O. The PCR conditions were within the following ranges: 94°C for 10 min, 30-35 cycles at 94°C for 30 s, 55°C for 1 min 15 s, 72°C for 1 min, and

72°C for 30 min. In the genome-wide scan of nine multiplex families performed in Finnish Genome Center, PCR reactions were done in 5µl volume containing 20 ng of DNA. Reagent concentrations and temperature profiles were as recommended by the manufacturer (Applied Biosystems, USA).

When screening the candidate regions in CPO families, the PCR products were fractionated on 6% polyacrylamide gel. The alleles were visualised by silver staining and they were numbered on the basis of their sizes. When screening 1q32-q41 and 17p11.2-p11.1 in VWS families, the allele sizes were separated on an ABI 377 laser fluorescent sequencing machine. In the genome-wide scan, the samples were electrophoresed on Megabace 1000 (Amersham Biosciences, http://www.moleculardynamics.com) 96 well capillary instrument according to the manufacturer's instructions. Allele calling was done using genetic Profiler 1.1 (Amersham Biosciences) software.

3.6 Linkage and LD analysis

For the linkage analysis in VWS families the penetrance was set to be 95 % (Sander et al. 1993), the model as autosomal dominant, the disease frequency as 1.5×10^{-5} (Rintala and Ranta 1981) and the mutation rate as 1.8×10^{-5} (Burdick et al. 1985). Individuals with CL, CL/P, CP and/or lip pits were considered to be affected. When analysing the modifying locus in 17p11.1-p11.2, the disease model was set as autosomal dominant with a penetrance of 70% and the disease frequency as 0.001 (Sertie et al. 1999). In this analysis, individuals with CP were classified as affected, regardless of the presence of lip pits. Multipoint linkage analysis was performed with the *Genehunter* program (Kruglyak et al. 1996).

For the linkage analysis of candidate region in non-syndromic CPO families no reliable parameters could be set. Therefore, we analysed non-random IBD allele sharing among affected individuals. This non-parametric linkage analysis was performed with the *Genehunter* program (Kruglyak et al. 1996). Pedigree no. 9 was too large for linkage analysis with *Genehunter* and was used for LD analysis only. Population-level association could be assumed and, therefore, we used transmission/disequilibrium tests to test for linkage with genetic markers (Spielman et al 1993). The TDT analysis was performed with the *Genehunter* software package.

In the genome-wide scan, the non-parametric linkage analysis was done using *Genehunter* 2.1. Pedigrees 1 and 9 were divided into two because of their size. The data were checked mendelian inconsistencies using Pedmanager and Pedcheck softwares before the linkage analysis.

3.7 Power estimations

The power to localize the disease gene by linkage disequilibrium was estimated with the help of computer simulations. One hundred data sets of 172 chromosomes were sampled from the multiplex CPO pedigrees. Random haplotypes *H* of different lengths 1, 2, and 3 were picked from each data set. Each of these haplotypes was enriched one at a time in the disease-associated chromosomes by replacing the corresponding alleles in each chromosome with haplotype *H* with probability *P* of 10%, 20%, and 30%. Probability *P* represents the extent to which an artificially introduced disease-associated haplotype is overrepresented in the affected sample. It is analogous to $P_{excess} = (P_{affected} - P_{normal}) / (1 - P_{normal})$, where $P_{affected}$ and P_{normal}

denote allele frequency in patient and control chromosomes, respectively. For each enriched data set, the highest χ^2 value was computed. Then, it was counted how often these highest values exceeded the corresponding critical thresholds for p=0.05 obtained from a permutation test (based on 100 iterations). This ratio corresponds to the power to detect linkage disequilibrium at type I error rate of 0.05.

3.8 COL2A1, COL11A1 and COL11A2 sequencing

DNA analysis

Genomic DNA was extracted from EDTA-preserved blood. Exons and exon-flanking sequences were amplified by PCR. Forty ng of DNA was amplified using 5 to 10 pmols of PCR primers, 1.5 mM of $MgCl_2$, 0.2 mM of dNTPs, and one unit of Amplitaq Gold DNA polymerase. The PCR conditions were as follows: 95 ° C for 10 min, 33 cycles at 95 ° C for 30 s, at 54-63° for 30 s, at 72° for 40 s, and finally at 72° for 10 min. Conformation sensitive gel electroforesis (CSGE) was used, and to generate heteroduplexes, the samples were denatured (95° C for 5 min) and re-annealed (68° C for 30 min). Twenty ng of each PCR product was analysed on CSGE gel. Samples that showed heteroduplexes on CSGE were sequenced (ABI Prism 377 Automatic Sequenator and BigDye Terminator Cycle Sequencing Kit, Applied Biosysterms). The DNA analysis, except for the extraction of DNA, was carried out in the Department of Medical Biochemistry and Molecular Biology, University of Oulu.

RNA analysis

RNA was extracted from EBV-transformed lymphoblasts. cDNA was synthesized and amplificated. The following PCR conditions were used: 95° C for 10 min, 33 cycles at 95° C for 40 s, at 60° C for 40 s, at 72° for 45, and finally at 72° for 10 min. Two µl of PCR product, 34 pmol of each primer and five units of AmpliTaq Gold polymerase were used, and the PCR products were analysed on 1.2% agarose gel. The products of the second PCR were then sequenced. The RNA analysis was also carried out in Oulu University.

RESULTS

4.1 Power estimations

The empirical power levels are summarized in Table 2. It shows that the power to detect linkage disequilibrium is adequate in most candidate regions, if the haplotype of at least two markers is moderately in excess (P at least 20%) in the affected sample.

L/P	**10%**	**20%**	**30%**	**L/P**	**10%**	**20%**	**30%**
1	0.14	0.39	0.67	**1**	0.09	0.29	0.47
2	0.35	0.79	0.94	**2**	0.17	0.61	0.96
3	0.36	0.92	1.00	**3**	0.23	0.90	1.00
2q32				**MSX1**			
L/P	**10%**	**20%**	**30%**	**L/P**	**10%**	**20%**	**30%**
1	0.17	0.57	0.87	**1**	0.03	0.15	0.41
2	0.46	0.96	1.00	**2**	0.07	0.49	0.76
				3	0.18	0.67	0.93
TGF-β3				**22q11**			

Table 2 (modified from the table 3 from the article number IV)**.** The empirical power levels to detect association in candidate regions. L denotes length (number of markers) and P denotes power to detect association when given % of chromosomes share the same genotype/haplotype.

4.2 Families with Van der Woude syndrome

Family 0062 was uninformative for all nine markers in the VWS region in 1q32-q41. Families 0059, 0060 and 0061 displayed linkage to 1q32-q41. Cumulative LOD scores obtained from these three families exceeded 3.8 across the VWS region. Families 0060, 0061 and 0062 shared a common haplotype 3 – 5 – 3 – 5 – 3 from markers D1S1663 to D1S3754. Linkage between VWS and 1q32-q41 was clearly excluded in family 0057, multipoint LOD scores ranging from -10 to -21. Also linkage between a proposed modifying locus in 17p11.2-11.1 and VWS was excluded. The cumulative LOD scores were less than –2. In this analysis, the results from family 0062 were disinconclusive.

Because linkage between 1q32-q41 and VWS in family 0057 was excluded, a genome wide search was performed. In the initial scan, a 30 cM region in chromosome 1p34 showed the highest LOD scores. The genotyping of additional markers in this region increased the maximum LOD score to 3.18 in marker D1S2797 with theta=0. The information content was 0.91. The LOD score of 3.18 is close to the theoretical maximum in this pedigree achieved by simulation using SLINK with 200 replicates (data not shown). Additional markers did not narrow the haplotype shared by all the affected. Changing the phenocopy rate to $1x10^{-3}$ had no effect on LOD scores. Lower penetrance of 90 % gave the maximum LOD score of 3.16. In all other regions of the genome, the LOD scores fell below 1.49.

4.3 Families with non-syndromic cleft palate

4.3.1 Candidate regions

Linkage between non-syndromic cleft palate and candidate loci could not be detected. In chromosome 2, the highest Z score (1.34, p=0.09, information content 0.61) was in locus D2S423. Markers in the candidate region 2q32 showed no evidence of linkage; the maximum Z score was 0.54 (p=0.29) and the information content was 0.78. In chromosome 22, the highest Z value was 1.36 (p=0.09) with the information content of 0.77. In the TGFβ3 region in chromosome 14, the Z score was 0.80 (p=0.20, information content 0.56). The MSX1 region in chromosome 4 showed negative Z scores. The highest Z value in chromosome 4 was 1.64 (p=0.06). Transmission/disequilibrium tests did not demonstrate any significant deviation from the expected values. No allelic association could be detected.

Marker D22S944 exhibited null alleles in 10 out of 24 families. Null alleles were carried by fourteen of the affected and 17 of the unaffected. The results were similar with a new set of primers. Individuals carrying null alleles seemed to be homozygous in that locus, but mendelian inheritance errors could be detected. No other mendelian errors occurred in chromosome 22. The marker D22S944 was checked in controls (28 Finnish SLE patients and 63 of their healthy relatives) and again similar null alleles were found in 6 patients and 5 of the unaffected.

4.3.2 Genome-wide screen

When genome-wide screening nine multiplex families (chromosomes 1-22) (stage 1), no significant linkage could be detected. The highest NPL scores of all chromosomes in the nine largest pedigrees are shown in Figures 5-6. The highest NPL score was seen in chromosome 1 at the position of 50 cM from 1pter (Z=2.06, p=0.033, information content 0.58). NPL scores reaching values over 1.5 were seen in chromosomes 2 (Z=1.97, p=0.038), 6 (Z=1.58, p=0.062), 11 (Z=1.60, p=0.061), 12 (Z=1.80, p=0.045) and 17 (Z=1.58, p= 0.062). Significant p-values (< 0.05) were seen in chromosomes 1, 2 and 12, at the positions of 50, 27 and 111 cM, respectively.

Stage two consisted of an analysis of candidate region 1p34 and entire chromosomes 2 and 4 with 24 multiplex families and with a denser marker map (~5 cM). When data from an additional 15 pedigrees (pedigrees 10-24) were added, the NPL score in chromosome 1 at the position of 50 cM fell to 1.31 (p=0.098). In the VWS-linked region in 1p34, the highest NPL score was 1.52 (p=0.069, information content 0.86) at the position of 61 cM. When screening the entire chromosomes 2 and 4 in 24 multiplex pedigrees, the highest NPL scores were 2.29 (p=0.016, information content 0.67) at the position of 27 cM, and 1.56 (p=0.064, inf. cont. 0.67) at the position of 100 cM. In chromosome 2, NPL scores over 1.5 (p<0.05) were seen at ~5-30 cM. In chromosome 4, the initial scan with nine families did not show any linkage but additional families and a denser marker map raised a narrow and shallow peak of 1.56 (p=0.064) at 100 cM. The NPL scores over the entire chromosomes 2 and 4 are shown in Figure 7. The NPL scores in the VWS-linked region in 1p34 are shown in Figure 7.

4.4 COL2A1, COL11A1 and COL11A2 sequence variations in patients with Robin sequence, non-syndromic micrognathia and CPO

Less than 20 % of variations detected in CSGE were in exons. Nine nucleotide substitutions cause one amino acid to chance to another, or creates a translation-termination codon. Six of these substitutions were found only in patients and in none of the 150 controls.

Two unrelated patients (35 and 39) with Robin sequence had a C to T transition in exon 17 in COL2A1. The mother of patient 39 was homozygous for the nucleotide chance. She has CPO. The RT-PCR of patient 35 did not show any splicing defect. Both alleles were expressed. The father of patient 35 also had the nucleotide change but he does not have any cleft.

Patient 26 with Robin sequence was found to carry an insertion of a T at the donor site of intron 50 at nucleotide position +3 in COL11A1. This variation has previously been reported in a patient with Marshall syndrome (Annunen et al. 1999). The parents of patient 26 did not have the mutation. Patient 22 with Robin sequence had a G to A transition in IVS45+3 in COL11A1, and the patient's mother carried the same mutation. This variation has also previously been reported in a patient with Marshall syndrome. Both alleles were expressed in this patient with Marshall syndrome. Two unrelated patients (20 and 33) with RS had a T to A transition in IVS31-92 in COL11A1. Both the patients have inherited the mutations from a parent who is healthy and without any cleft.

Patient 62 with Robin sequence was found to have a C to T transition in exon 4 in COL11A2. This mutation changes Arg to translation termination. The patient's father had the same nucleotide change and he was reported to have a high arched palate and small upturned nose. Patient 53 with non-syndromic micrognathia had a C to T change in exon 13 in COL11A2.

DISCUSSION

5.1 About the aim of the study

Cleft palate is one of the most common congenital malformations. Its incidence varies among and between races but, in practise, it is seen worldwide. Treatment of a cleft patient demands a highly specialised team of professionals. Surgical reconstruction of cleft palate requires special technical skills. Because of secondary problems (ear infections, speech delay, growth disturbances of facial bones, dental problems) related to oral clefts, patients must be regularly followed up during their growing period. Thus, cleft palate causes a medical and social burden to the patient and financial costs to the society. Perhaps in the future, gene therapy can be applied also to orofacial clefts, but it should be emphasized that there are many open questions in gene therapy, and that gene transfer has not yet been used as evidence-based treatment in any disease.

A family with a child with an oral cleft, should always be given an opportunity to meet a clinical geneticist. This meeting should have two purposes: First of all, the family should be informed about the mechanisms and the genetics of clefts. A very important goal of this information is to vanish false assumptions and guilt. Secondly, an estimation of the recurrence rate should be given. To give this estimation, the physician should rule out cleft syndromes, which are inherited dominantly or recessively. To rule out these syndromes, the child and also, if necessary, other family members, should be carefully examined. One of the aims of our study was to obtain new information about the mechanisms and the genetics of cleft palate to be given to the families.

5.2 Methodological considerations

Cleft palate is considered to be a genetically complex disease. In addition to genetic factors, there are extrinsic factors that influence cleft formation. In non-syndromic CPO, there is no clear mendelian model for inheritance. Studies on cleft syndromes have taught us that defects in functionally very different types of genes can cause cleft palate. Because of these facts, we have been obliged to pay particular attention to phenotyping and choosing the right method for the linkage analysis. These problems are common to all gene-mapping projects concerning complex, multifactorial diseases (Lander and Schork 1994, Risch and Merikangas 1996)

The decision to choose patients with CPO and exclude patients with CL and CL/P was based on previous observations on the epidemiology and embryology of oral clefts. A cleft of the primary palate and the lip (one or both sides) can occur together with a cleft of the secondary palate. However, if a proband has a cleft of the secondary palate, the recurrence risk of a cleft of the primary palate for relatives is no higher than the population risk, and vice versa (Fogh-Andersen 1942). This observation, combined with the observation on different timing in embryonic development (see above) leads us to assume that there might be partially different etiological factors causing and disturbing either the closure of the primary palate only or the secondary palate only. This assumption is also strengthened by the fact that there are syndromes in which a cleft palate is a constant sign but a cleft lip is practically never seen. One should still keep in mind that in some patients these two forms of cleft occur simultaneously, and that there exist syndromes in which both forms can be seen separately in a particular pedigree.

Among CPO patients, it can not be assumed that all affected individuals with similar CPO phenotypes carry the same mutation, or that they carry mutations in a particular gene. There are several problems to be considered when working on genetically complex, multifactorial diseases (Schork et al. 1997). In locus heterogeneity, defects in any gene can lead to a disease independently of each other. Classical polygenic inheritance denotes a situation in which a number of mutations in different genes must be transmitted to an individual before they result in disease. In epistasis, the combined effect of two genes is "more" than or "different" from the sum of their separate effects (Frankel et al. 1996). The time-dependent expression of a gene has its most pronounced deleterious effect at a particular developmental stage. Extrinsic factors can modulate gene expression. Any combination of these variations in gene action can exist in cleft palate formation.

This fact inevitably leads to the question: Do we have enough power to find linkage or LD (if one or both exist) in our study? Our simulations showed that in most candidate regions the power to detect linkage disequilibrium is adequate, if the haplotype of at least two markers is moderately in excess (P at least 20%) in the affected sample.

The other problem is that the penetrance and the model for inheritance of CPO are not known. Therefore, we chose to use non-parametric linkage analysis when screening genome-wide and candidate regions. Sib-pair analysis does not require prior assumptions on parameters but, unfortunately, there are not enough affected sib-pairs with CPO in Finland. CPO has a relatively large λ value and, thus, distant relatives offer greater power than sib pairs (Risch et al. 1990).

5.3 VWS

Previously, all the VWS families have shown linkage to 1q32-q41 (Murray et al. 1990, Schutte et al. 1996, Sertie et al. 1999, Wong et al. 1999, Houdayer et al. 1999). We found a large Finnish family to be unlinked to 1q32-q41. It is remarkable that one affected individual only carried a lip pit. The diagnosis was based on this sign. Usually lip pits are seen in ~80 % of patients. In this family, cleft palate was seen in all but one of the affected, and only one of the affected carried cleft lip and palate. This phenomenon can reflect the predomination of CP over CL/P in Finland (Lilius 1992). Other modifying loci or possibly extrinsic factors might be involved in determining the exact phenotype. It can also be speculated whether these two observations might imply a new subtype or variation of the Van der Woude syndrome.

In our study we found VWS in this large family to be linked to 1p34. Several interesting candidate genes map to 1p34. CRTM (cartilage matrix protein, matrilin-1) has already been excluded as a major gene in non-syndromic CL/P families (Vintiner et al. 1993). Endothelin-A receptor–deficient mice manifest cranial neural crest defects (Clouthier et al. 1998). Endothelin is essential for normal postmigratory differentiation in neural crest (Maschhoff et Baldwin 2000). Endothelin 2 maps to the region implicated in family 0057. Multiple epiphyseal dysplasia is caused by mutations in COL9A2 (Muragaki et al 1996), and cleft palate is seen in many skeletal dysplasias. The new finding on mutations in IRF6 causing VWS will teach us a lot about cleft formation in the near future.

In family 0062 the haplotypes of the father were IBS (3-5-3-5-3) in VWS region. Analysis of extended haplotypes showed that the first affected child has inherited the maternal haplotype from her father and the other affected child has inherited the paternal haplotype from his

father. Both the parents of the father are unaffected. No clefts are known the have existed in previous generations or in distant relatives. Interestingly, this haplotype is found also in two other pedigrees with VWS (0060 and 0061) in affected members. It might be possible that the father of family 0062 is a true homozygous for VWS causing mutation in 1q32-q41.

5.4 Non-syndromic cleft palate

On the basis of our study, it is obvious that 22q11, 2q32, MSX1 or TGF-β3 do not have major roles in cleft palate formation in our study sample of the Finnish population. Although no linkage nor association could be detected, these genes and genes in these regions might still have some influence on cleft formation.

The genome-wide scan did not find any significant linkage. However, several points should be emphasized. In the initial scan with nine pedigrees, the highest NPL scores were seen in chromosome 1p in the same region (48-52 cM, $p<0.05$), which was linked to VWS in pedigree 0057 (39-89 cM, $p<0.0005$). With fifteen additional families (10-24) and six additional markers in 1p34, the NPL scores fell below 1.52 ($p=0.069$). In chromosome 2 at ~5-30 cM (2p24-p25) we found a suggestive linkage ($Z>1.5$ ($p<0.05$)), Z_{max} being 2.29 ($p=0.016$). This is the first time it has been reported that 2p24-p25 shows any suggestive linkage to CPO. An interesting region was also found in chromosome 12 at 111.0 cM (12q21) ($Z=1.80$, $p<0.05$). Marazita et al. had previously found significant associations for two loci in chromosome 12 but in different locations (78 and 166 cM).

5.5 Robin sequence

Robin sequence is a triad of cleft palate, micrognathia and glossoptosis (Robin P 1923). However, there is no convincing evidence that it is a true sequence. The high incidence of twins among RS patients supports the theory that lack of space mechanically induces RS. On the other hand, a large proportion of RS patients have either Stickler or velocardiofacial syndrome, and this fact shows that well-known mutations can lead to RS. Recent conclusions suggest that RS is causally heterogeneous and also pathogenetically and phenotypically variable (Cohen 1999).

In our study, we wanted to check whether mutations in genes involved in Stickler sdr cause Robin sequence, non-syndromic micrognathia or non-syndromic cleft palate. Two unrelated patient with non-syndromic RS had a similar mutation in exon 17 in COL2A1. The mother of the other patient was homozygous for the mutation. She has cleft palate only. The mutation does not change the encoded amino acid. No splicing defects could be detected and both alleles were expressed. Thus, it is unlikely that this nucleotide change would be disease-causing, although it was not seen in controls. Three different nucleotide changes, which were not seen in controls, were detected in COL11A1 in patients with non-syndromic RS. Two of these mutations have previously been described in patients with Marshall syndrome. Our patients did not show any clinical features suggesting Marshall syndrome, but different phenotypes have been described in other diseases caused by collagen defects. Nucleotide changes IVS45+3G>A and IVS50+3insT were now reported for the first time in patients with non-syndromic RS. A nucleotide change IVS31+92T>A was detected in two patients with non-syndromic RS. Both these patients had inherited the substitution from an unaffected parent. All these three nucleotide changes in COL11A1 are likely to be disease-associated in non-syndromic RS. An American patient with non-syndromic RS carried, in exon 4 in

COL11A2, a mutation which changes a codon for arginine to a codon for translation termination. It is likely that no functional protein is producted by the mutated allele. A patient with non-syndromic micrognathia carried, in exon 13 in COL11A2, a mutation which converts arginine to trp. This change should also have some effect because trp is not found in the non-triple helical region between the major and minor triple helical domains in fibrillar collagens.

We found several interesting nucleotide changes in COL2A1, COL11A1 and COL11A2, mainly in patients with RS. They are not clearly disease-causing but disease-associated. The etiopathology of Robin sequence needs further studies.

CONCLUSIONS

Three different sequence variations in COL11A1 were found in four out of 24 non-syndromic RS patients. Two of these variations have previously been reported in patients with Marshall syndrome. Two different sequence variations in COL11A2 were detected, one in a RS patient and the other in a patient with micrognathia. An identical variation in COL2A1 was detected in two unrelated patients with RS. None of these sequence variations were seen in controls. We conclude that these sequence variations can have impact on RS, CPO and micrognathia in some but not in majority of these patients.

We studied five Finnish VWS families and in four of them VWS was linked to previously reported VWS locus in 1q32-q41. Linkage to 1q32-q41 was clearly excluded in one large pedigree. When, analysing this pedigree, we found in a genome-wide scan a second locus for VWS in 1p34, which has not previously been reported.

Candidate regions 2q32 or 22q11 and candidate genes MSX1 or TGFβ3 do not play major roles in cleft palate formation in Finnish families with non-syndromic cleft palate. Genome-wide scan in these large, multiplex families did not reveal any significant linkage although several interesting regions were found. The most interesting region is in 1p34 (48-52 cM), which is the same region that VWS in pedigree 0057 with was linked (39-89 cM). We conclude that in non-syndromic cleft palate there is locus heterogeneity, but there is a strong evidence that in some families –yet an unknown- gene in 1p34 has impact on cleft formation. This region in 1p34 needs to be studied further on.

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Helsinki, November 2003

FIGURES

Figures 1-3. Pedigrees 1-24 of families with CPO.

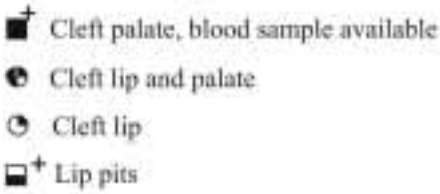


Figure 4. Pedigrees of VWS families.

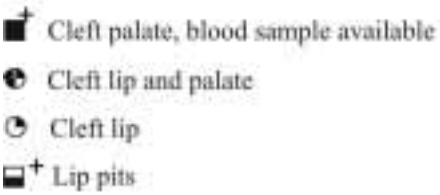
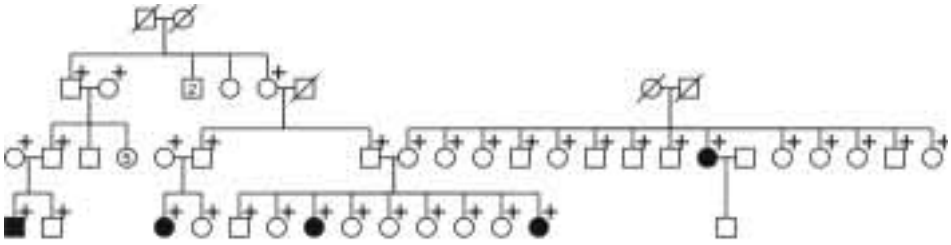


Figure 5. NPL scores and information content of chromosomes 1-15 in nine multiplex families (1-9).

Figure 6. NPL scores and information content of chromosomes 16-22 in nine multiplex families (1-9).

Figure 7. NPL scores and information content of 1p34, chromosomes 2 and 4 with denser markermaps in all 24 multiplex families.



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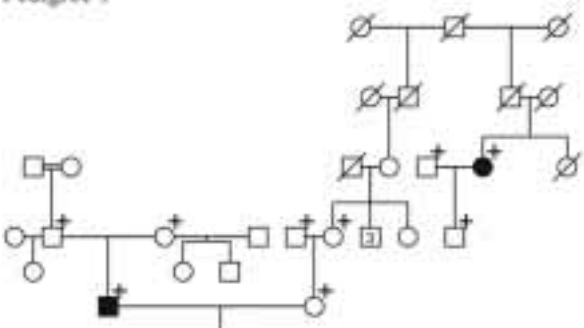

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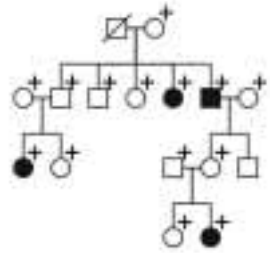

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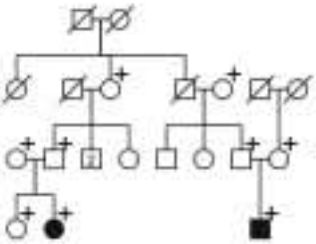

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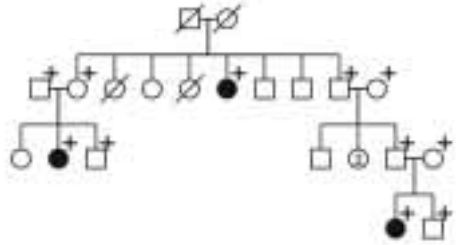
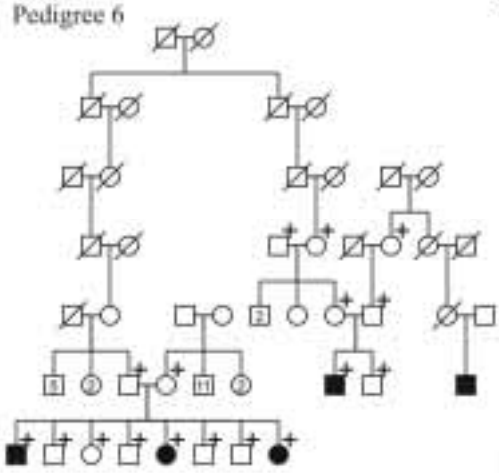


Pedigree 5

Pedigree 6



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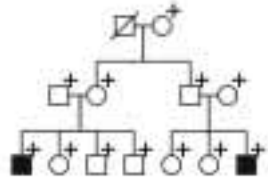

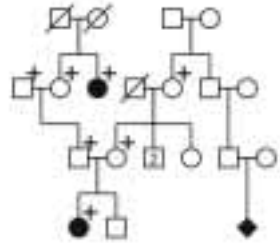

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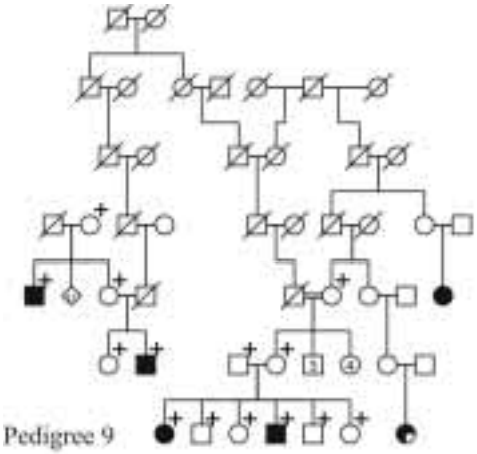

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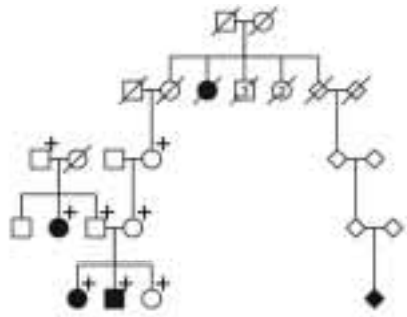

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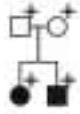

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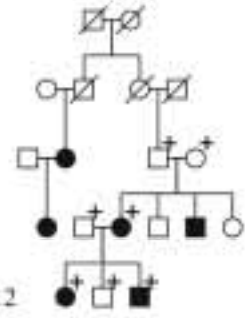

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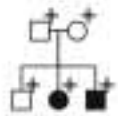

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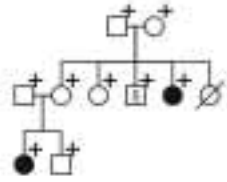

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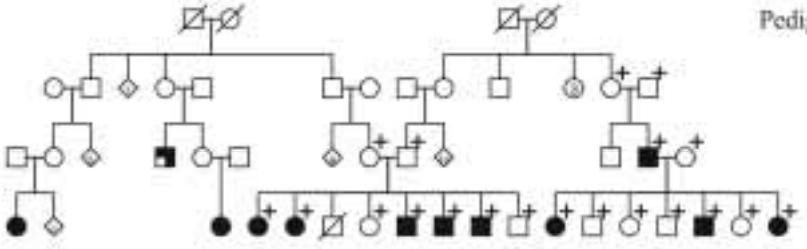
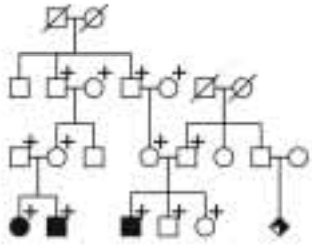


Pedigree 13

Pedigree 16



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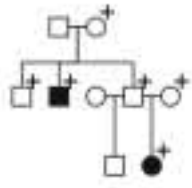

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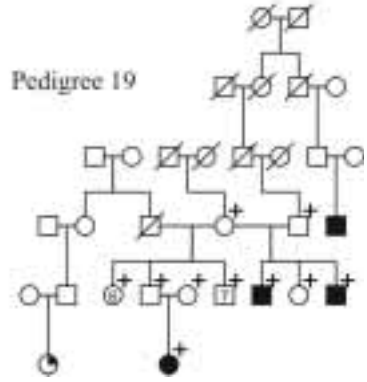

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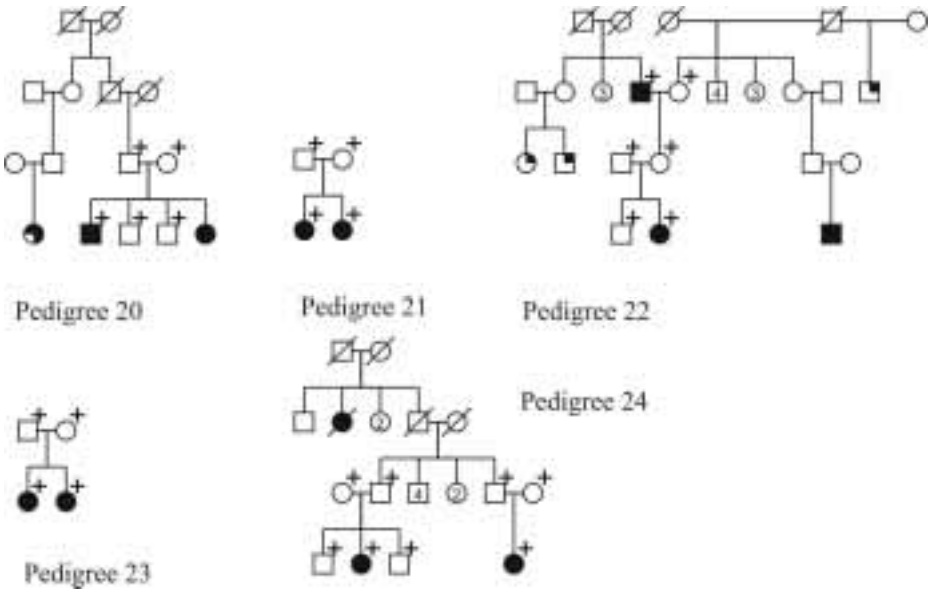


Figure 3.

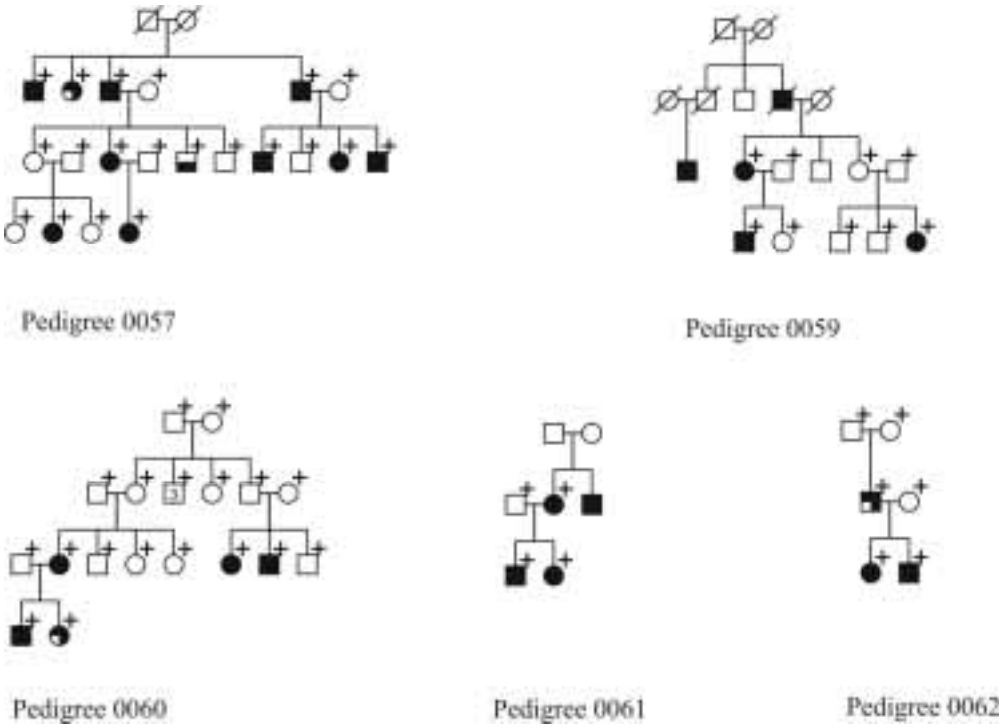
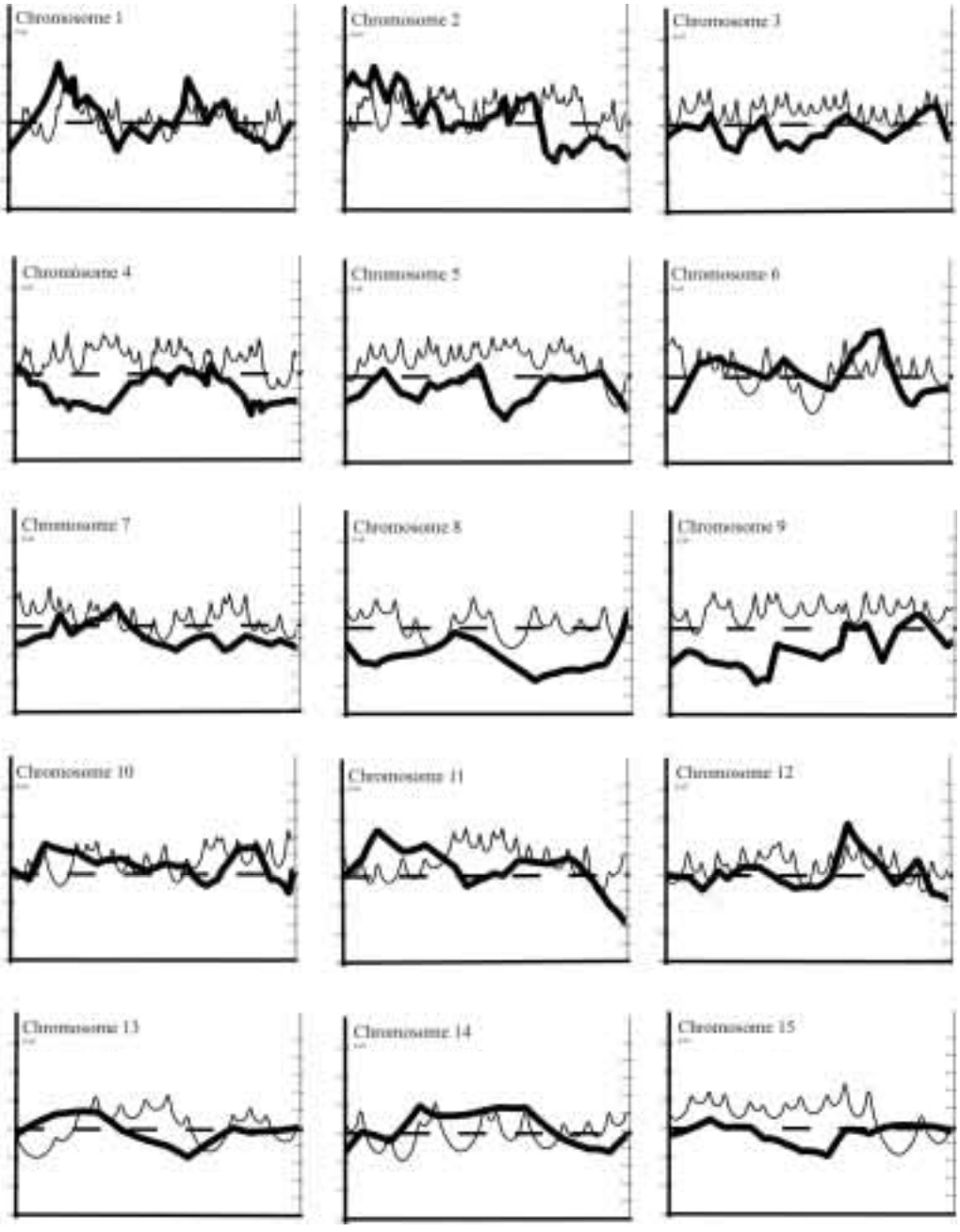


Figure 4.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Chromosome 2
Chromosome 3
Chromosome 4
Chromosome 5
Chromosome 6
Chromosome 7
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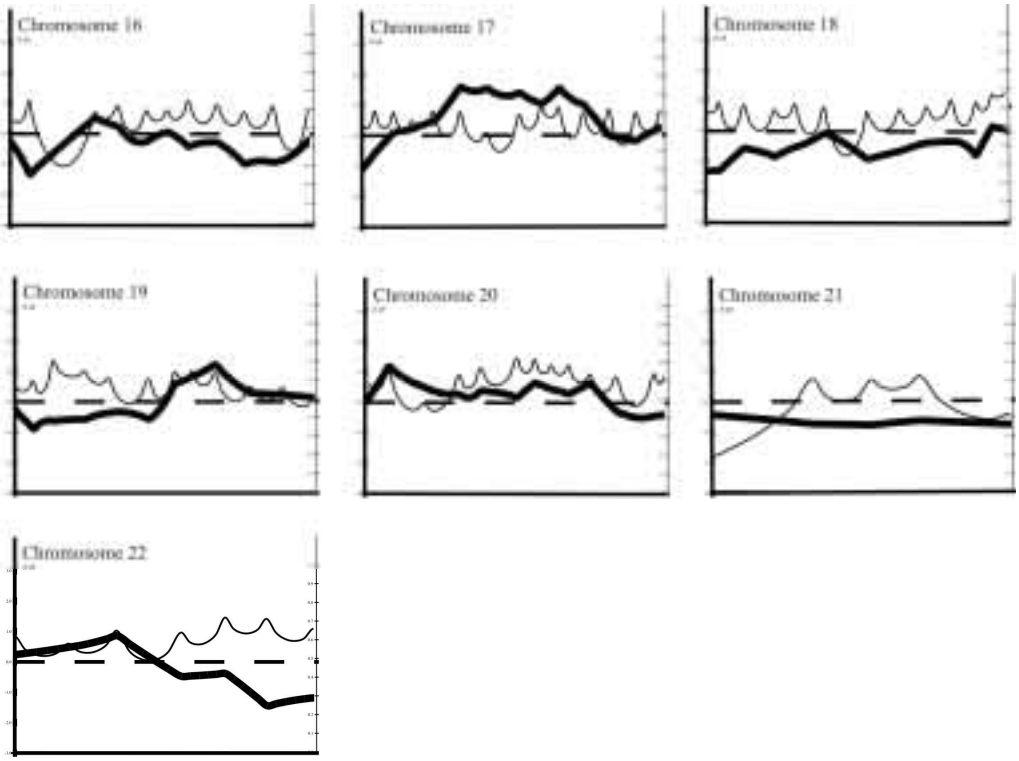
Chromosome 11
Chromosome 12
Chromosome 13
Chromosome 14
Chromosome 15

Figure 5.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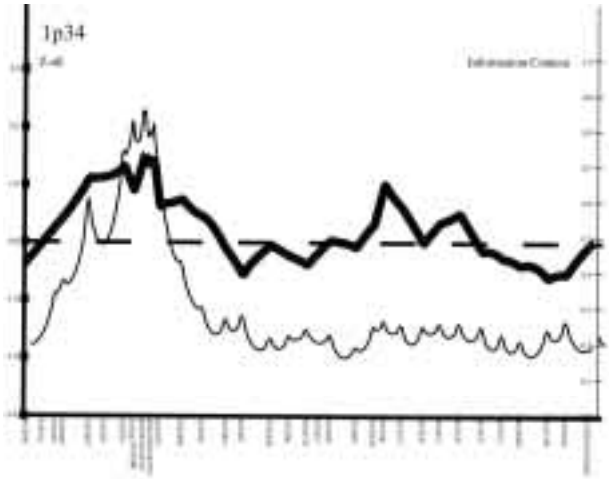
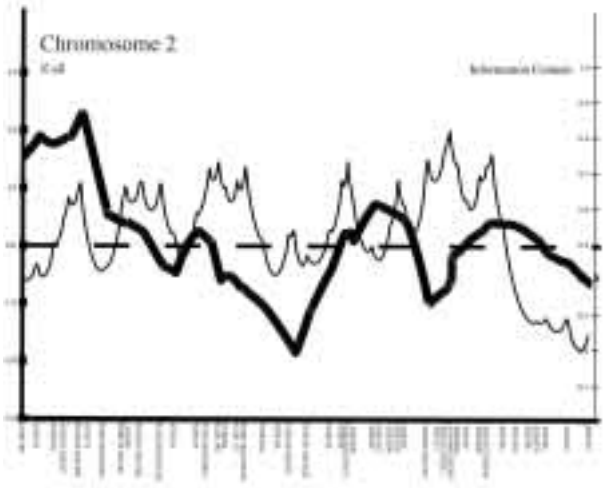
Chromosome 17
Chromosome 18
Chromosome 19
Chromosome 20
Chromosome 21
Chromosome 22

Figure 6.


1p34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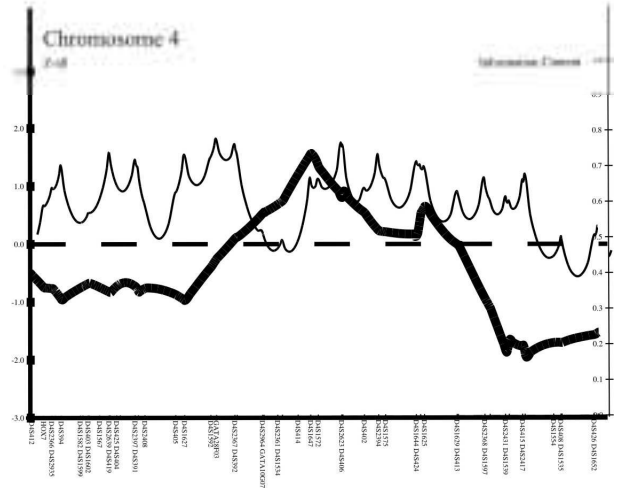
Chromosome 4
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1.0
0.0
-1.0
-2.0
-3.0
0.8
0.7
0.6
0.4
0.3
0.2
0.1

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