Ph.D. Thesis by Dorthe Grosen, MD

Oral Cleft Occurrence and Familial Aggregation among Danish Twins and Singletons

70 Years of Follow-up

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This PhD Thesis is based on the following three papers:

Paper I

Grosen D, Chevrier C, Skytthe A, Bille C, Mølsted K, Sivertsen A, Murray JC, Christensen K. **A cohort study of recurrence patterns among more than 54,000 relatives of oral cleft cases in Denmark: support for the multifactorial threshold model of inheritance.**

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Paper II

Grosen D, Bille C, Pedersen JK, Skytthe A, Murray JC, Christensen K. **Recurrence Risk for Offspring of Twins Discordant for Oral Cleft – A Population-based Cohort Study of the Danish 1936-2004 Cleft Twin Cohort.**

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Paper III

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Abbreviations

A	Additive genetic variance	PIN	Personal identification number
C	Shared environmental variance	PPR	Prevalence proportion ratio
CI	Confidence Interval	Prefix i	Isolated/non-syndromic
CL	Cleft lip	RR	Relative risk
CLP	Cleft lip with cleft palate	SES	Socioeconomic status
CL(P)	Cleft lip with or without cleft palate	ss	Same sex
CP	Cleft palate only	UZ	Unknown zygosity
C_{Pr}	Probandwise concordance rates		
CRS	The Civil Registration System		
D	Non-additive genetic variance		
DFCD	The Danish Facial Cleft Database		
DF	Degrees of freedom		
DTR	The Danish Twin Registry		
DST	Statistics Denmark		
DZ	Dizygotic		
E	non-shared environmental variance		
GxE	Gene-environment interaction		
GWAS	Genome wide association studies		
MZ	Monozygotic		
OC	Oral cleft		
OR	Odds ratio		
os	Opposite sex		

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1. Introduction

Cleft lip and palate - also called oral clefts (OCs) - are among the most frequent congenital malformations with about 140 new cases every year in Denmark out of approximately 62,000 births[1]. The OC occurrence vary with geographic origin and socioeconomic status[2]. The individuals affected by this structural anomaly require several surgical procedures early in life. In childhood and youth corrective procedures on the teeth, nose, and jaw are done and logopedic assistance is crucial for development of normal speech. Danish studies have shown that later in life individuals with an OC have an increased overall mortality[3], an increased risk of psychiatric diseases[4], and an increased risk of breast cancer has been indicated[5].

Non-syndromic oral clefts are complex traits since they exhibit no classical Mendelian inheritance, but show strong familial aggregation and have a substantial genetic component[6-9]. For the majority of individuals affected by OC, the etiology is still unknown and no larger intervention is known to reduce OC occurrence.

Familial aggregation and inheritance of OC can be studied by use of family and twin studies. Valid results are, however, dependent on high ascertainment of a large unbiased sample of OC cases with sufficient power to detect even a modest association.

The first one to exploit this area of research in Denmark was Dr. Poul Fogh-Andersen in his thesis "Inheritance of Harelip and Cleft Palate" in 1942[10], followed by Dr.'s Kaare Christensen and Camilla Bille. Their work has already answered several important questions concerning familial aggregation and twin occurrence, but with a recent update of the Danish Facial Cleft Database (1936 -2005)[1] a substantial improvement of the precision of prior estimates as well as new analytical opportunities were provided.

The Danish Facial Cleft Database can be linked to the Danish Twin Registry (1870-2004) by use of the Danish Civil Registration System. This thesis employs these three registers in order to study the OC occurrence and familial aggregation for twins and singletons. The isolated OC recurrence risk for first, second and third degree relatives is estimated with high precision (paper I). The recurrence risk pattern is explored in greater detail for the first degree relatives to test the premise for the multifactorial threshold model of inheritance for isolated OC since the model has been challenged by a recent Norwegian study using a smaller sample (paper I). The isolated OC recurrence risk for offspring of twins discordant for OC is estimated (paper II). Finally, it is determined whether twinning is associated with isolated OC and the relative contribution of genetic and environmental factors to the OC etiology is provided (paper III).

A discussion of the impact of this new evidence, pooled with the existing world literature and future perspectives, will be the closing of this thesis.

1.1. Embryology

The lip and the palate are formed in the first trimester of the pregnancy when the shelves of the primary and the secondary palate fuse. The fusion is a complex mechanism where each step is controlled by the expression or suppression of various genes of importance for the embryogenesis. A disturbance of this closely controlled mechanism can hinder the normal fusion of the lip and the palate so that the child is born with an OC.

The upper lip, the maxillary dental alveolus, and the primary palate fuse during the 4th and the 7th gestational weeks and the secondary palate fuse between the 8th and the 12th gestational weeks. Since the primary palate develops before the secondary palate, improper fusion of the primary palate, leading to a cleft lip (CL), can cause the shelves of the secondary palate to be malpositioned such that the palatal shelf contact and fusion may not occur resulting in a cleft lip with cleft palate (CLP). Embryological are CL and CLP therefore closer related than the CL/CLP are with cleft palate only (CP).

1.2. Clinical features of oral cleft

1.2.1. Oral cleft phenotype

The classification of overt OCs can be based on either pathogenesis or etiology. Based on the anatomic/embryological considerations the OCs can be divided into three main groups: 1. Clefts of the lip and primary palate (CL); 2. Clefts of the primary and secondary palate (CLP); and 3. Clefts of the secondary palate (CP). CL can range in severity from a small notch in the upper lip (incomplete) to a complete opening of the lip extending into the bottom of the nose. The upper gum (primary palate) is most often involved. CP is a cleft in the secondary palate, and it is always median. CP can involve only the soft palate (in the back of the mouth) or extend forward through the hard palate. The mildest form of CP is a cleft underneath the mucosa only (sub-mucous CP). The clefts of the lip and primary palate can be either uni- or bilateral, with the unilateral being the most common, with a left preponderance. The distribution between the phenotypes is approximately 1/3 to each. A graphical figure of the overt cleft phenotypes is shown in figure 1.

Figure 1. Oral cleft classification. Blue color: Primary palate, Pink color: Secondary palate. A: Cleft lip. B: Unilateral cleft lip and palate. C: Bilateral cleft lip and palate. D: Cleft palate.
Graphics by Susanne Kløjgaard.
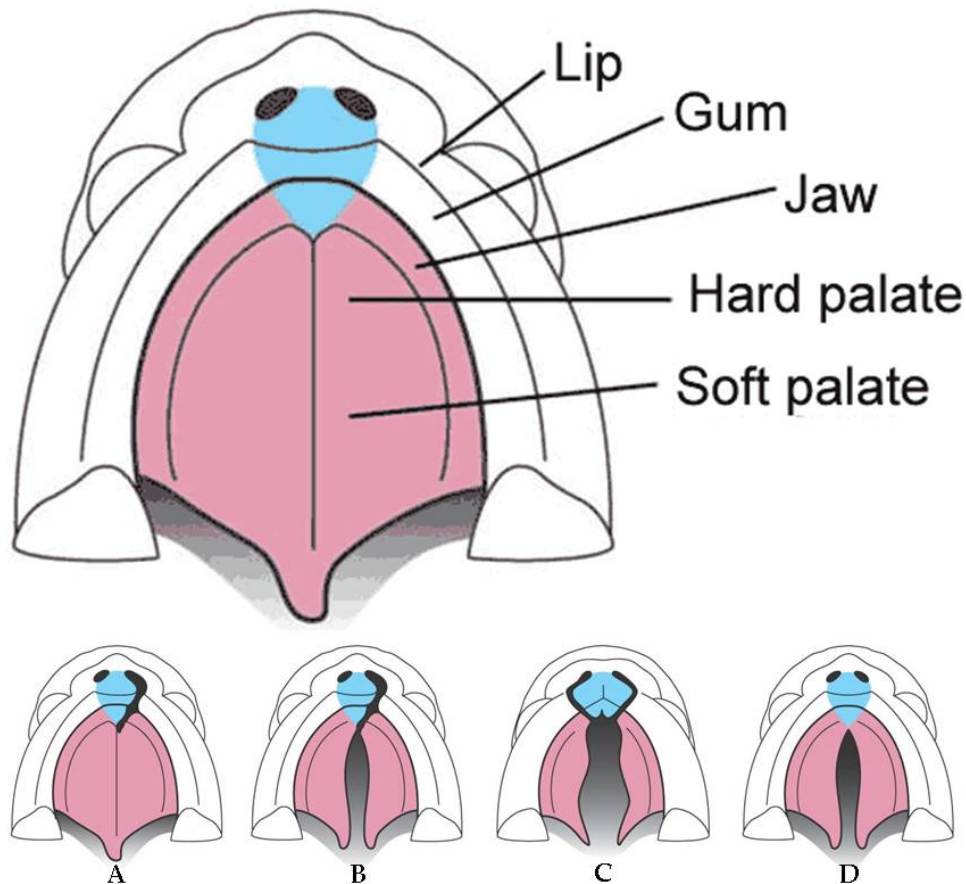
Etiological arguments can be found for the same classification. Fogh-Andersen, 1942, was the first to establish that CL(P) and CP are two etiologically distinct phenotypes[10]. CL and CLP may also be etiologically distinct or may represent a continuum of severity with CLP being the more severe form of the defect[11]. Either way, CL and CLP should, when possible, be evaluated separately[12]. In rare cases (likely by chance) and in syndromic forms of oral clefting, all three phenotypes are found in the same family.

Until recently, the overt OC phenotype has been thought of as a qualitative trait (affected or unaffected). However, an increasing number of studies have shown that the OC phenotypes are more complicated and may be characterized by a variety of associated subclinical markers (termed sub-phenotypes) or endophenotypes, that are seen in either individuals with clefts and/or their non-cleft relatives. For example, an increased frequency of disruptions of the orbicularis oris muscle, dental anomalies, or other adverse facial morphologies are found among relatives of OC individuals compared to the general

population[13-19]. A broadening of the OC phenotype to include also the hidden clefts would lead to identification of more individuals at increased risk of carrying cleft genes. This would enhance the chance of identifying these genes of importance in the OC etiology so that the genetic counseling could be personalized. Aggregated the OC phenotype will, however, still be dichotomous, but the criteria for diagnosing an individual with OC will have changed and the different types might be categorical or, possibly, ordinal.

1.2.2. Associated syndromes/anomalies

OCs can be associated with other major physical or developmental anomalies and/or may be a part of a recognized syndrome. In these cases, the OCs are classified as a syndromic clefts as opposed to isolated or non-syndromic clefts. A wide range of the frequency of syndromic clefts has been reported in the literature: 10-30% for CL(P) and 20-60% for CP[20-22]. Previous Danish studies reports approximately 10% of CL(P) and 30% of CP associated with a syndrome or a major anomaly but the estimates are vulnerable to ascertainment bias[1]. These numbers are lower than in other populations, but the pattern with more anomalies/syndromes associated with CP compared to CL(P) is the same[20;21]. The syndromic OCs can be seen as part of the more than 400 single-gene Mendelian syndromes (Online Mendelian Inheritance in Man, 2010), as part of chromosomal abnormalities, or as part of syndromes due to teratogenic exposures.

1.3. Occurrence

OCs are among the most common birth defects and its occurrence depends on ethnicity and gender. Native Americans and infants of Asian descent have the highest prevalence of CL(P) followed by Caucasians. The lowest prevalence is seen for infants of African descent[23;24]. The CP prevalence is less sensitive to ethnical influences[24;25]. More boys than girls are born with OCs varying with the phenotypes. There is a male predominance for CL(P) and a female predominance for CP[10;26].

With a birth prevalence of one in 500, Denmark joins the countries with the highest prevalence[1]. The Danish prevalence has been stable for the last 65 years despite massive changes in lifestyle factors, indicating little impact from the environment on the OC etiology[1;27]. In other populations both a decreasing (England/Wales) and an increasing (Finland/Norway) trend have been noted however sensitive to ascertainment methods[24].

1.4. Etiology

Since Poul Fogh-Andersen first identified genetic factors in the clefting etiology[10], it has become increasingly apparent that the genetic contribution to OC is complex, probably heterogeneous, and likely due to interacting loci coupled to environmental covariates. OCs

may be caused by genetic derangements, the influence of teratogens disrupting the normal developmental process, or mechanical forces interfering with the normal tissue formation.

1.4.1. Genetics of oral cleft

Before the actual search for genes of importance began in the 1980s, a line of studies elucidating the possible effect of genes on the OC etiology had been carried out. Family and twin studies serve this purpose well, but many challenges must be met when using family and twin studies of OCs: (1) The familial aggregation depends on ascertainment of relatives over a long period of time in order to provide valid estimates of recurrence for first, second and third degree relatives. (2) Even though OCs are relatively common, the co-occurrence with twinning is rare. (3) The need of a long ascertainment period combined with relatively rare conditions (OC and twinning) makes it difficult to obtain an unbiased sample of OC cases with sufficient power to detect even a modest association. These problems are aggravated since (4) the syndromic forms of oral clefting should be excluded and stratification should be done for (5) OC phenotypes and (6) zygosity.

1.4.1.1. Familial aggregation and mode of inheritance

A positive family history is the most consistently identified risk factor for OC measured by recurrence risk studies. These studies measure the risk of OC occurrence in a subsequent child if it has appeared already once in a nuclear family. The definition can be broadened to include all types of first, second and third degree relatives in a family if data are available. On the Danish 1952 to 1987 cohorts, recurrence risks for first, second and third degree relatives were provided. For CL(P) the recurrence risks were 3.2%, 0.06%, and 0.1%, respectively, and for CP 2.7%, 0.3%, and 0%, respectively[6,7]. For offspring of twins discordant for OC, no empirical data of a sufficient size have previously been available. A hypothesis was based on case reports from 1996 and 2002 [28,29] concerning one family of OC discordant twins. The authors speculated that the risk for the offspring of the unaffected co-twin in the pair would be three to ten times higher than the risk of the background population, i.e. potentially as high a risk as for the affected twin, but likely to be smaller, since the co-twin was unaffected.

The magnitude of the family recurrence risk can be an indication of the strength of a genetic contribution, and the pattern of recurrence can indicate a mode of inheritance. Knowledge of mode of inheritance allows for more accurate genetic counseling and is widely used when designing studies aimed at identifying disease-causing or pre-disposing loci[12,30]. A modification to a simple Mendelian inheritance invoked by Fogh-Andersen in 1942[10] cannot explain that the concordance rates for monozygotic twins are less than 100%, that the decline in recurrence risk with decreasing degree of genetic relationship to the probands is nonlinear, and that recurrence risks are dependent on severity[31]. Since the early 1950s, clinical practice has been to counsel parents of a child

born with a cleft on the risk of having a subsequent child with an oral cleft using empiric recurrence risks consistent with the multifactorial threshold model of inheritance[12;31-33]. The multifactorial threshold model provides a theoretical framework for the complex interactions of multiple genes and the environment.

The hallmarks for multifactorial inheritance are: 1. Most affected children have normal parents, 2. Recurrence risk increases with the number of affected children in a family, 3. Recurrence risk increases with severity of the defect, 4. Consanguinity slightly increases the risk for an affected child, 5. Risk of affected relatives falls off very quickly with the degree of relationship and, 6. When the two sexes have a different probability of being affected, the least likely sex, if affected, is the most likely sex to produce an affected offspring[34].

This model has been challenged by several complex segregation analysis studies but there has not been sufficient evidence to reject the model[35;36]. Since the 1990s several studies of both recurrence patterns as well as the identification of specific loci or genes contributing to clefts have ruled out a single, major locus model and the multiplicative, additive, or independent loci models. Ultimately, the best fitting model of inheritance is multiple genes interacting with each other and/or environmental factors which agrees with the multifactorial threshold model[6;7;37-43]. Analyses have suggested that there are likely to be two to 14 genes (possibly more but most likely three to six) interacting multiplicatively involved in the etiology of CL(P)[6;39;44]. A recent study using a single, well-defined population from Norway has challenged the multifactorial threshold model since they found no effect of severity on inheritance[45]. If this result can be replicated in additional and larger studies it would have substantial implications for the clinical counseling of families and the understanding of the underlying causes of clefting[46].

1.4.1.2.   Twins studies

Two central questions for twin studies of OCs are:

1. Is there a special etiology for twins with OC compared to singletons?

2. What information can the twins provide about the genetic contribution to OC etiology for the general population of, predominantly singleton, affected individuals?

The answers are intertwined since a yes to the first question will render the information obtained about the genetic contribution of little value for the general population of affected individuals.

Several studies have compared the OC occurrence in twins and singletons. The results are ambiguous and most studies are limited by small sample size, ascertainment bias, inclusion of syndromic forms of OC, and a lack of zygosity information[47-57]. So far, the

majority of data have not provided compelling evidence of OC to be associated with twinning in general or with monozygotic (MZ) twinning in particular[8;9;58-61].

When estimating the relative contribution of genetic and environmental factors to the OC etiology, the basic assumptions about twins are utilized. The MZ twins share 100% of their genes as opposed to the dizygotic (DZ) twins who, on average, share 50% of the parental genetic pool, but the shared environment is perfectly correlated within both MZ twin pairs and DZ twin pairs. Any differences in the concordance rates between the MZ and DZ twins must therefore be attributable to their differences in genetic similarity. The probandwise concordance rate (C_{Pr}) has previously been estimated on a small Danish twin population born from 1970-1990[8;9]. The C_{Pr} for MZ twins was about 60% and between 0 to 10% for DZ twins with corresponding heritability estimates of approximately 70%. However, the estimates were hampered by the small sample size[26] especially for CP. The large difference between the C_{Pr} for MZ and DZ twins provided evidence that genetic variation underlies the phenotypic expression of OC. Nevertheless, less than 100% phenotypic concordance indicates that environmental factors e.g. smoking could be of importance since the genomic sequence alone cannot explain the disease susceptibility. The environmental factors may, however, not sufficiently explain the MZ twin discordance, which could also result form epigenetic phenomena such as X inactivation or DNA methylation[62]. Another explanation could be reduced penetrance or chance. Finally, discordant MZ twins could also arise from somatic genetic events such that the affected twin might be the only member of the pair carrying a specific risk allele[63-65]. Since the C_{Pr} is an estimator of the probability that one twin has an OC given that the co-twin is affected, it can be directly compared to the recurrence risk for singleton siblings, who are genetically equivalent to DZ co-twins[66]. This comparison offers the possibility to single out the effect of the environment since the number of shared genes is similar for DZ twins and non-twin siblings, but the twins shared the uterus while the siblings were there at different points in time. A change in the environment could either be an intentional change in the mothers risk behavior in the subsequent pregnancy after having a first child with OC, or a change in other environmental factors that the mother was not in control over. It has not been possible either in the Danish population or in any other population to determine whether DZ twins have an excess risk of OC compared with singleton siblings.

1.4.1.3.   Genes of importance

There are in general now four approaches in gene mapping: chromosomal anomaly studies, linkage studies, association studies and direct sequencing. Chromosomal anomalies, particularly the small deletion/duplication events now found by array technology, can identify candidate regions or genes when an event is detected in a case with OC. Examples of success with this approach include the *SUMO1* gene [67;68]. *Linkage*

studies identify a disease locus through co-segregation of a known genetic marker with the disease phenotype. Linkage studies are done on families and can focus on candidate genes/regions or be genome wide and not dependent on an a priori hypothesis. *Genetic association studies* measure the non-random association of a specific genetic marker allele(s) with a phenotypic trait. A direct association exists when the genetic marker studied in itself plays a causal role in the disease phenotype. An indirect association is one in which the genetic marker is located in proximity to the causal variant on the genome (linkage disequilibrium), and hence an association can be shown. Association studies can be carried out on a group of cases compared to healthy controls or on a collection of nuclear families (the parents' untransmitted alleles can serve as controls for the affected child and/or transmission disequilibrium of risk alleles from a heterozygous parent to an affected child can be examined). Candidate gene association studies are dependent on a priori knowledge about the candidate genes as opposed to *genome wide association studies* (GWAS) that can be carried out with no a priori hypothesis. Candidate genes can be selected from those genes known to cause syndromic forms of clefting, genes expressed in the relevant craniofacial tissues during embryogenesis, or from results from linkage or expression studies or knockout models on mice.

Since 1985 large scale studies have been performed in the search for genes of importance for OC. The initial efforts relied mainly on candidate gene approaches. This method has identified several genetic associations with OC[69;70]. The *FOXE1* gene was found to be associated with OC by use of *fine-mapping* of a region identified by a genome-wide linkage study [71]. Linkage studies, however, work best for monogenic diseases like the syndromic form of OC, Van der Woude syndrome. For isolated OCs the results have merely attested to the locus heterogeneity of OC[72]. In 2009 the first results from a GWAS were published, and a region at chromosome 8q24 was found to be associated with OC[73;74]. This result has been replicated in two other GWAS, and other new risk variants have been identified (found at loci adjacent to the genes *MAFB*, *ABCA4*, and *VAX1*)[75;76].

The use of the described methods in the study of *syndromic forms of OCs* has facilitated significant findings regarding the etiology of isolated OC. Linkage studies have led to the identification of a region on chromosome one resulting in Van der Woude syndrome - a dominantly inherited syndrome expressing all OC phenotypes along with lip pits and dental anomalies. A pair of MZ twins discordant for Van der Woude syndrome led to the identification of a mutation in the *interferon regulatory factor 6 (IRF6)* gene on chromosome one causing Van der Woude syndrome using sequencing[64]. Further studies have led to the identification of other variants at the *IRF6* gene also associated with isolated OCs[69;77]. The *IRF6* gene has shown the largest degree of consistency across studies[78;79]. This promising discordant MZ twin design has been

applied to isolated oral clefting, but with no success so far[62;63;80]. Examples of other genes involved in syndromic forms of OC, that also contribute to isolated OC found through candidate gene sequencing, are mutations in the FGF signaling pathway[81], *MSX1*[82-84], *PVRL1*[85-87], and *TBX22*[88]. With the onset of massive parallel sequencing it is now possible to contemplate whole exome or even whole genome sequencing to find variants for clefting as it has already been done successfully for several Mendelian disorders[89-91].

When the results from the genetic studies are summed up, a crude estimate of the attributable risk suggests that, at best, the known genetic variants contribute to about 25% of the isolated OC cases[92]. While this is a significant step forward, the biological consequence of most of these genetic variants is yet to be unraveled and more genes are to be identified. GWAS, and most likely *next-generation sequencing,* might provide these advances, but linkage studies using loaded families are still of value when searching for rare variants.

1.4.2. Environmental factors (teratogens)

Despite indications of environmental factors having a lesser impact on the OC risk than the genetic factors, research is ongoing and important because of the potential immediate prevention prospects. When studying environmental factors, the magnitude of the association, the consistency of results in several studies, measures of dose-response relationships and a biological plausibility must be taken into account.

1.4.2.1. Smoking

Smoking is known to be associated with a line of adverse pregnancy outcomes and oral clefting is no exception. The first studies looked at the association with aggregated congenital birth defects[93]. Since then numerous case control studies and a few prospective studies on the subject have been published. A few studies could not identify a positive association between smoking and OC[94;95], but the majority of studies have provided evidence for an association with either one or both of the OC phenotypes (CL(P) and CP)[96-102]. To improve the sample size and add power to the estimates, a meta-analysis was conducted. The relative risks (RRs) were modest but highly significant, 1.34 (95% CI 1.25 to 1.44) and 1.22 (95% CI 1.10 to 1.35) for CL(P) and CP, respectively[103]. The RR for smoking corresponds to an attributable risk of about 4% for all OCs and 12% for CL(P)[102].

1.4.2.2. Alcohol

Alcohol is a human teratogen that produces a line of effects depending on the timing of the exposure and the amount of alcohol consumed[104]. OCs can be associated with fetal

alcohol syndrome, the most severe alcohol related outcome[105]. This association has prompted an intense search for an association between alcohol and isolated OCs. The results are conflicting likely due to differences in ascertaining and classifying individuals, measuring the amount, timing of the exposure, recall bias and evaluating confounders[106-112]. Most studies reported an association between alcohol consumption and CL(P) (odds ratio (OR) (≥ five drinks per day) = 3.0, CI 1.1 to 8.5)[106]. Only few studies observed a significant association between alcohol and CP (OR=3.0, CI 1.4 to 6.5)[111], which is likely due to smaller samples of CP individuals. Few have reported a dose-response association[108]. The majority of studies were case control studies. A Danish study, however, was a prospective study that provided evidence for a harmful effect of alcohol but with wide confidence intervals[101].

Binge drinking (more than five drinks per sitting), rather than the regular use of alcohol while pregnant, has in both animal models and human studies been suggested to be the most relevant alcohol measurement for assessing potential adverse fetal outcomes[113]. Werler et al found a significant association between binge drinking and CL(P), OR=3.0, 95% CI 1.1 to 8.5 [106]. This finding has been corroborated for CL(P) in another study where similar effects of binge drinking on the risk of CP (OR=2.6, 95% CI 1.2 to 5.6) were observed[114]. Larger prospective studies are still needed to be able to replicate or refute this hypothesis.

1.4.2.3.   Vitamins

An association between poor nutrition and the risk of OC has been suspected, but poor nutrition is a vague description, hence other specified studies have been carried out. The majority of interest has been focused on folic acid, but vitamin A and various vitamins Bs have also been studied.

1.4.2.3.1. Folic acid

Folate is essential for the synthesis of nucleic acids (DNA and RNA), involved in the synthesis of certain amino acids and necessary for the normal detoxification processes within the liver. The interest in folic acid and OC was intensified after a randomized clinical trial showed a protective effect of folic acid intake in the first trimester on the risk of neural tube defect[115;116]. The evidence that folate deficiency increases the risk of CL(P) measured by the protective effect of folic acid supplement is robust, but for CP the results are ambiguous[117-120]. In a meta-analysis five prospective studies yielded combined RRs of 0.51 (95% CI 0.32 to 0.92) for CL(P) and 1.19 (95% CI 0.43 to 3.28) for CP, and 12 case-control studies provided combined RRs of 0.77 (95% CI 0.65 to 0.90) for CL(P) and 0.80 (95% CI 0.69 to 0.93) for CP[118]. A recent Norwegian case-control study showed a clear tendency of the higher doses being the most protective[119].

Despite the World Health Organization's recommendation of a 400 μg folic acid supplementation a day, less than 50% of the women follow the recommendation and a high rate of unplanned pregnancies compound this problem[121;122]. Several studies have shown a reduction in both neural tube defects and OCs after a mandatory fortification of stable foods like wheat, corn flour, or rice has been implemented[123-125]. In Denmark and in most other European countries, the governments have been hesitant to implement mandatory fortification with folic acid, since the relatively narrow target group (women getting pregnant) may not justify an intervention at population level when there is a risk of masking pernicious anemia and indications of an increased cancer risk[126], among others[127].

1.4.2.3.2. Other vitamins

For other vitamins like A-vitamin (retinoic acid), B-2, B-6, and B-12 vitamins animal studies have provided evidence for an association with OC along with a biological plausibility[128;129]. Some studies on humans have confirmed this indication[130-133], but the total number of studies are few and not all, including a Danis study, could corroborate the findings[101]. The strongest evidence has been provided for retinoic acid indicating a protective effect in therapeutic doses, and a possible harmful effect in toxic doses[134-136].

1.4.2.4. Medication

In general, when studying the effect of drugs an adverse pregnancy outcome, confounding by indication should be taken into account. For example, when studying the effect of antiepileptic drugs on the OC risk, the pregnant women with epilepsy in need of medication may differ from the non-medicated epileptic pregnant women in other aspects confounding the results. It could also be the epilepsy itself resulting in the malformation instead of the drugs taken[137]. Many types of drugs have been studied, but only for antiepileptic drugs has an association with OC been shown repeatedly[138-140]. The highest risk was seen when more types of drugs were used[141]. Antiepileptic drugs are of lesser public health importance, but the effect is of large significance for the individuals in need of the medication.

1.4.2.5. Other exposures

Increasing evidence has been provided for an association between low socioeconomic status and the risk of OC[142;143].

Furthermore, indications have been made of organic solvents and agricultural chemicals[138;144;145], stress[146], coffee[147], increased parental age[148], and increased BMI[149-151] having an effect on the risk of OC. Larger studies are needed to either confirm or refute these findings.

1.4.3. Gene-gene and gene-environment interaction

The influence of the environment and genetic variants on the OC etiology is compounded by gene-gene (GxG) and gene-environment (GxE) interactions. Since the formation of the lip and the palate is under close genetic control, having mutations in one or more genes it seems plausible that those genes could interact in a multiplicative manner and increase the risk of OC further. Statistical evidence for such a GxG interaction leading to OC has been reported for *MSX1* interacting with *TGFB3*[152] and *MSX1* with *TGFA*[153]. The mechanism is thought to be a reduced function of *MSX1* combined with a reduced function of *TGFB3* or *TGFA* which can lead to CLP. It may, however, also be a genetic variation of the mother, combined with a genetic variation in the fetus that could increase the risk of OC. Such indication has been made for the maternal *MTHFR* genotype interacting with the infant's *BCL3* genotype[154], but it has not been replicated.

When looking at the GxE interaction it is known that the environmental agents interact with the maternal gene products, but whether that also happens for fetal gene products is not clear. However, it seems plausible that a fetus may have a low risk for OC due to its genes, but that the risk increases due to the mother's environmental exposures and her genetic susceptibility to these exposures. Studies involving the maternal effects on OC risk have focused exactly on genes involved in either detoxification or maternal folate intake; hence the MTHFR, an important enzyme in the folate metabolism, has been intensely studied. No such maternal interaction between a variation in *MTHFR* genotype and the risk of OC could be shown in a South American population[155], but a significant GxE interaction between the infants *MTHFR* genotype and the mothers folic acid consumption was shown[156;157]. A similar interaction has been reported between smoking and fetal *GSTT1* resulting in a nearly 20-fold increased risk of CLP if the mother smoked more than 15 cigarettes per day[158]. This finding could, however, not be reproduced on a French population[159]. For alcohol has an association with *ADH1C* been shown [160] as it has also been shown for fetal *NAT1* and either maternal smoking or lack of multivitamin intake[161;162]. A suggested interactions between smoking and *TGFA* could, however, not be replicated on a Danish population[96;163].

In summary, attributable fraction estimates suggests that known genetic variants contribute to about 25% of the isolated OCs, and that smoking contributes to approximately 5%[92]. An effect of antiepileptic drugs has also been provided, but the public health impact is small. Furthermore, increasing evidence supports an association between OC occurrence and low socioeconomic status, and a protective effect of folic acid on the risk of OC. Even so, the etiology is still largely unknown with no effective intervention to reduce OC occurrence. If the information about the OC genes identified is combined with the information obtained about the familial aggregation, genetic counseling can be improved. Moreover, if in future studies of the effect of environmental factors

on the OC risk the effect of the known genes could be controlled for and interactions identified, the possibilities of identifying environmental factors with a prospect of intervention would also be improved.

2. Aim

The overarching aim of this thesis was to describe the OC occurrence and familial aggregation among Danish twins and singletons in order to provide estimates for genetic counseling and improve the understanding of the OC etiology.

The specific aims were:

- To estimate the OC recurrence risk for first, second and third degree relatives of individuals affected by an OC (*paper I*).

- To test whether the premise for the multifactorial threshold model of inheritance was fulfilled for isolated OC by stratifying the OC recurrence risk for first degree relatives by severity, specificity, parent of origin effect and family size (*paper I*).

- To estimate the OC recurrence risk for offspring of unaffected twins in twin pairs discordant for OC and to compare this risk to the risk of the affected twins and the background population (*paper II*).

- To estimate the effect of twinning on the risk of isolated OC by comparing the OC occurrence among twins and singletons (*paper III*).

- To estimate the nature (i.e. additive versus non-additive) and magnitude (i.e. heritability) of the genetic influences on the OC etiology (*paper III*).

- To compare the probandwise concordance rates for DZ twins to the OC recurrence risk for non-twin siblings (*paper III*).

3. Material and methods

3.1.   Data sources

Denmark holds a long tradition for collecting data into well established nationwide population and health registries. In Denmark the healthcare is free, ensuring that most patients will use governmental health care resources, which maximizes ascertainment of all kinds of healthcare events including OC. In the present thesis individuals registered in three of these extensive registries have been linked (figure 3) and aggregated data from a fourth register have been exploited.

Figure 2. Cohorts ascertained in the Danish Civil Registration System, the Danish Twin Registry and the Danish Facial Cleft Database.
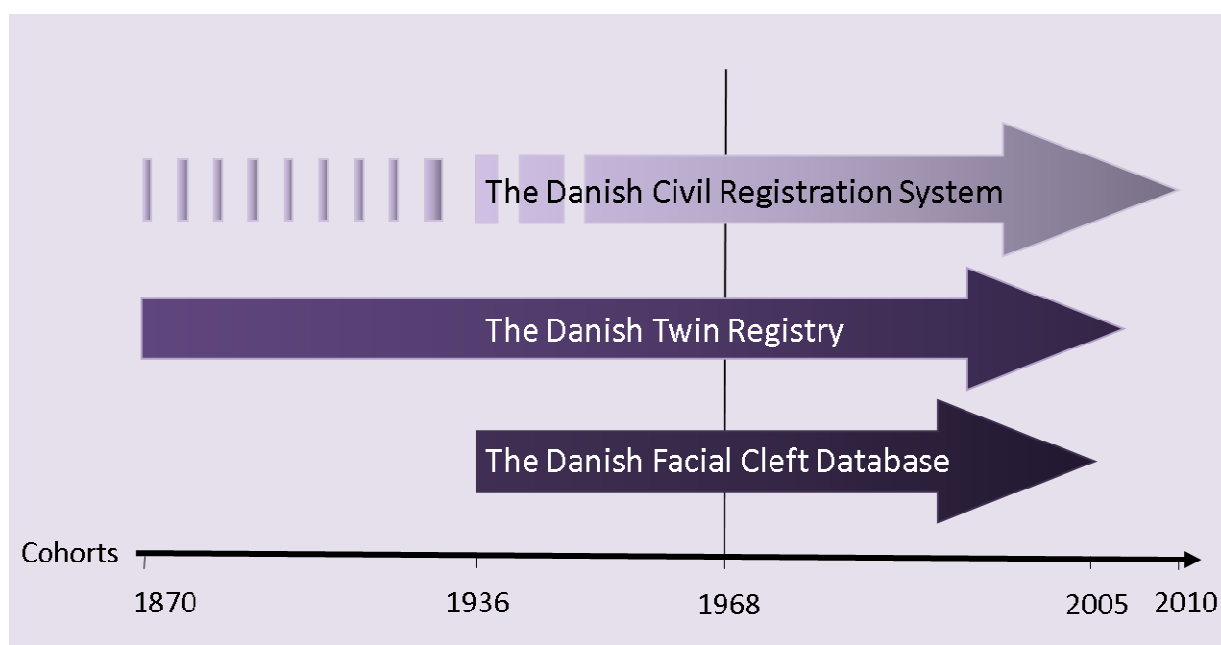
3.1.1.   The Danish Civil Registration System

The Danish Civil Registration System (CRS) was established April 2nd, 1968, and it has registered all individuals alive and residing in Denmark since then for administrative purposes[164]. All individuals have a unique ten-digit personal identification number (PIN), where the first six digits disclose the date of birth, the following three digits is a serial number, and the last digit denotes the sex (even and uneven numbers indicate female and male, respectively). Additionally, the CRS contains complete information on name, place of birth and current residence, date of death, and migration status. This register also includes identifiers that link all first degree relatives (parents and siblings). These identifiers allow construction of sibships (by matching individuals with parental PINs) which can be linked using parent sibships to form complex pedigrees. On the maternal side links have been

almost complete (96%) since 1959, but for individuals born before 1952, it is considerably lower (46%). A similar pattern is apparent for the paternal PINs although the availability tends to be slightly lower (92% post-1959 and 39% pre-1952).

3.1.2.   The Danish Facial Cleft Database

The Danish Facial Cleft Database (DFCD) now encompasses the 1936 to 2005 cohort. It includes 10,025 live born individuals born with an OC of whom 9,146 (91.2%) individuals are registered by a PIN and 231 of them are twins. Two nationwide ascertainment sources have been used to ascertain the Danish individuals with OC.

1. *The Deaconess Hospital and the University Hospital of Copenhagen*. For the earliest birth cohorts all patients were operated by Dr. Poul Fogh-Andersen at the Deaconess Hospital. He maintained a careful list of all patients he operated from 1934 to 1986. Since then, all surgery has been and still are performed at the University Hospital of Copenhagen.

2. *The two National Institutes for Defects of Speech*. Since 1954, midwifes in Denmark have been obliged to report all individuals born with an OC to these two institutes, where all treatment other than surgical may occur. OCs, mainly sub-mucous CP, recognized later in a child's life are also reported to the institutes.

The ascertainment is high for the complete cohort, and capture-recapture methods have indicated 99% ascertainment for the sub-phenotype isolated CL(P) in the period 1983 to 1987[165]. Individuals born in Greenland and the Faroe Islands are not included in the DFCD. In the DFCD overt OCs are classified into three groups, i.e. CL, CLP, and CP. Both cleft of the lip only and cleft of the lip and the primary palate are considered a CL phenotype. A distinction with regards to the completeness of the CL is not possible in the DFCD, but a record is made of whether the cleft is unilateral or bilateral. For CP, three sub-classifications can be identified: sub-mucous CP, cleft in the soft palate only, and cleft in the hard and soft palate. Bifid uvula is considered a microform of CP, but is not routinely registered in the DFCD. Despite the increasing evidence for a series of other structural changes in the face and skull, like dental anomalies and defects of the orbicularis oris muscle being microforms of OCs[14-17;19], they are not registered in the DFCD.

In the DFCD 876 (9.6%), individuals born with an OC are registered as having at least one additional major anomaly or a recognized syndrome. Malformations such as neural tube defects were designated as major anomalies. Defects such as polydactyly were considered minor malformations. Minimal defects such as nevi were not considered associated anomalies. The determination of whether the associated anomalies was considered minor or major has been consistent in the DFCD since the inception of the registry[26]. The determination is based on whether the anomaly is likely to be part of a syndrome. For the earlier birth cohorts from 1936 to 1987, the number of individuals born with either an associated major anomaly or a syndrome was likely underestimated[165], but for the later

birth cohorts medical records were reviewed by Bille et al. in 2005 to obtain more complete information about associated anomalies/syndromes[1,5].

3.1.3.   The Danish Twin Registry

The Danish Twin Registry (DTR) comprises more than 80,000 twin pairs born in Denmark since 1870. The twins are ascertained independently of any disease. The information about the oldest cohorts was collected from birth registries in each parish in the Kingdom of Denmark. A local clergy was asked to report twin births of which both twins had survived to the age of six. With the establishment of the CRS, the ascertainment improved markedly and registration at birth became possible. The overall ascertainment of live born twins from 1930 and onwards was about 80%, since 1968 the ascertainment has been considered complete for live born twins, and since 1973 it has been complete for all twins[166]. Zygosity determination of same sex pairs has been made based on four standard questions about physical resemblance, a method with less than 5% misclassification for the birth cohorts 1900-1982[167]. Zygosity determination on twins with OC was made using the same method, also with a misclassification estimated to be less than 5%[8,168]. About 75% of the twins in the register have an assigned zygosity. Information on zygosity is only accessible through the DTR.

3.1.4.   Statistics Denmark

Statistics Denmark (DST) comprises information about many aspects of life for all residents of Denmark. This information has been collected for administrative purposes on a consistent basis since the establishment of the CRS. Since then, it has been possible to track individuals by use of the PIN, but before 1968 the data were aggregated[169]. Several thematically organized databases are kept within DST. Different registers span different time periods, and it is the length and completeness of this coverage which is important for the retrospective measurement of background and outcome information. The complete DST database relates to the whole population of Denmark - about 5.4 million persons. From this database, the number of twins born from 1936 to 2005 has been drawn.

3.2.   Statistics

For the present thesis, the OC population was restricted to all live born individuals with a valid PIN in the CRS.

Different subsets of the cohorts available in the DFCD were used in the three papers. In paper I the cohorts born between 1952 and 2005 registered with isolated OC were included. Individuals born before 1952 were excluded since their records in the CRS were unlikely to include parental links. An exception was made for the grandparents of the probands so that grandparents born from 1936 to 2005 were included, but only if the intervening parent was

born between 1952 and 2005. In paper II the 1936 to 2004 OC twin cohorts were used since only a link to offspring was needed reducing the concerns of introducing bias. When comparing the OC occurrence between twins and singletons in paper III, the 1936 to 2004 cohorts were included. When stratified for zygosity only the younger cohorts from 1968 to 2004 were used.

Operationally, the probands from the DFCD were first linked to the CRS using their PIN. Because the CRS allowed the identification of first degree relatives for each proband, the total number of affected and unaffected relatives of each phenotype could be counted. The DTR was linked to the DFCD in order to identify twin pairs of whom at least one of the twins was affected with an OC, and hence the OC twin population was identified.

All recurrence risks were estimated by dividing the number of affected relatives of type R (R = parents, offspring, etc.) by the total number of relatives R. The recurrence risk for later born siblings and for full siblings according to family size was also estimated. Probands and siblings who were members of a twin pair were not included in sibling estimates. For all other types of relatives, twins were included as both probands and relatives in order to keep the groups as comparable as possible. For offspring of the unaffected twin in the OC discordant twin pairs, the same method was used when computing a pseudo-recurrence risk, even though an OC could technically not recur for an unaffected twin. The relative recurrence risks were estimated by dividing the recurrence risks with the population frequency of OC[6;7].

For first degree relatives the recurrence risks were provided for different degrees of severity, for the same or dissimilar types of isolated OCs, according to family size, and with respect to parent of origin effect. The bilateral clefts were graded as more severe than unilateral for both CLP and for CL only. For the CP cases, sub-mucous CP was graded as the mildest form and involvement of both the hard and soft palate as the most severe form.

OC prevalence and prevalence proportion ratio (PPR) for twins versus singletons stratified for sex and the sub-phenotypes: CL, CLP, and CP were estimated using summary data from DST[169]. When stratified for zygosity the data were linked to the DTR.

The relative contribution of genes and the environment to the OC etiology was estimated by use of both the C_{Pr}, the tetrachoric correlation (corresponding to the intraclass correlation for a continuous outcome), and heritability for CL(P) and CP. The C_{Pr} is estimated as two times the number of concordant affected pairs (both twins are affected) divided by two times the number of concordant pairs plus the number of discordant pairs (one twin affected). Tetrachoric correlations for MZ and DZ twins (same and opposite sex) were compared under the assumption of the multifactorial threshold model. A higher correlation for MZ twins compared to DZ twins indicates that genetic factors contribute to the phenotypic variation. The magnitude of the genetic contribution can be computed using heritability estimates which are independent of the prevalence of the malformation studied. For the tetrachoric correlations and the heritability estimates both same sex and opposite sex twin pairs were included, but

thresholds were not adjusted for effects of sex. The total variance *(V)* could be decomposed in *V* = *A* + *D* + *C* + *E* where *A* refers to the additive genetic effects, *D* refers to the dominant genetic effect (intraloci interaction), *C* refers to shared environmental effects (contribute to twin similarity) and *E* refers to the unique environmental effect (contribute to twin dissimilarity). Univariate genetic models[170] were fitted to contingency tables using maximum likelihood estimation with Mx statistical modelling[171]. First a saturated model was fitted and thereafter the following models were fitted: ACE, ADE, AE, CE and E. The best fitting model was chosen in accordance with the lowest Akaike Information Criterion (AIC) (χ^2-2·df). Thereby both the goodness of fit and the simplicity of the model were taken into account. The 95% confidence intervals (CI) were calculated for the standardized parameter estimates (heritability) of the best fitting model.

Further details about the statistics can be found in the three enclosed papers.

4. Results

4.1.   Familial aggregation (Paper I)

A total of 3,703,337 live births were registered in Denmark during the period 1952 to 2005. The analyses were carried out on 2,116 isolated CL probands, 2,572 isolated CLP probands, and 2,088 isolated CP only probands.

The observed recurrence risks (absolute and RR (λ)) for relatives of individuals affected by CL, CLP, or CP are shown in table 1. The recurrence risk for siblings of the CLP probands was estimated to 3.9% (95% CI 3.2% to 4.7%), and it was comparable to the estimate for the later-born siblings of 4.6% (3.5% to 5.8%). The risk of CLP for the offspring was 4.1% (3.2% to 5.1%) and also similar to the risk for the siblings. The risk to parents, however, was 2.5% (1.8% to 3.1%); this was significantly lower than the risk to either of the two other groups of first degree relatives. The RR of CLP for all first degree relatives was 17 (95% confidence interval 15 to 19) times higher than the risk observed in the background population.

The recurrence risk for second degree relatives (half siblings, nieces/nephews, aunts/uncles, and grandparents) were lower than the risk to first degree relatives and yet quite similar to each other. The RR of CLP for second degree relatives was 4 (3 to 5) times higher than the risk observed in the background population.

For third degree relatives (first cousins, half-nieces/nephews, and half-aunts/uncles) the recurrence risk was lower than the risk to second degree relatives and they were quite similar to each other. The risks of CLP for third degree relatives were 3 (2 to 4) times higher than the risk observed in the background population.

The same pattern was found for the other two cleft types for all three kinds of relatives.

For first cousins in particular the recurrence risk estimates for the three cleft types were indistinguishable (table 1). The overall estimate of the recurrence risk for OC for first cousins was 0.4% (95% confidence interval 0.3% to 0.6%), i.e. 2 (1.5 to 2.7) times higher than in the background population.

For all OCs aggregated, the recurrence risk for first, second, and third degree relatives was 3.1% (560/17,906), 0.7% (160/21937), and 0.5% (69/14,386), respectively.

Table 1: Risks of oral cleft for first, second, and third degree relatives according to the probands' three phenotypes of cleft (Denmark, 1952-2005)

	First degree relatives					Second degree relatives						Third degree relatives			
	Offspring	Siblings†		Parents	ALL	Half-siblings†		Nieces/ nephews	Aunts/ uncles	Grand-parents‡	ALL	First cousins	Half nieces/ nephews	Half aunts/ uncles	ALL
		All	Subsequent			All	Subsequent								
iCL probands (n=2,116)															
Total number	1,439	2,442	1,162	1,861	5,742	810	362	2,155	2,213	1,729	6,907	3,532	544	468	4,544
No. affected*	50	60	25	47	157	8	3	20	13	4	45	11	3	1	15
Risk (%)	**3.5**	**2.5**	**2.2**	**2.5**	**2.7**	**1.0**	**0.8**	**0.9**	**0.6**	**0.2**	**0.7**	**0.3**	**0.6**	**0.2**	**0.3**
Relative Risk (λ)	17	12	10	12	13	5	4	5	3	1	3	2	3	1	1.6
(95% confidence interval)	(13 to 22)	(9 to 15)	(7 to 15)	(9 to 16)	(11 to 15)	(2 to 9)	(0.8 to 10)	(3 to 7)	(2 to 5)	(0 to 2)	(2 to 4)	(0.8 to 3)	(0.5 to 7)	(0.0 to 4)	(0.9 to 3)
iCLP probands (n=2,572)															
Total number.	1,591	2,954	1,389	2,209	6,754	938	454	2,702	2,742	2,093	8,475	4,303	649	547	5,499
No. affected*	65	116	64	55	236	5	3	22	29	10	66	22	6	5	33
Risk (%)	**4.1**	**3.9**	**4.6**	**2.5**	**3.5**	**0.5**	**0.7**	**0.8**	**1.1**	**0.5**	**0.8**	**0.5**	**0.9**	**0.9**	**0.6**
Relative Risk (λ)	20	19	22	12	17	3	3	4	5	2	4	2	4	4	3
(95% confidence interval)	(15 to 25)	(16 to 23)	(17 to 28)	(9 to 16)	(15 to 19)	(0.8 to 5)	(0.6 to 8)	(2 to 6)	(3 to 7)	(1.1 to 4)	(3 to 5)	(1.6 to 4)	(2 to 9)	(1.4 to 9)	(2 to 4)
iCP probands (n=2,088)															
Total number	1,211	2,379	1,171	1,820	5,410	828	396	2,002	2,175	1,550	6,555	3,344	478	521	4,343
No. affected*	51	78	39	38	167	8	5	22	12	7	49	15	2	4	21
Risk (%)	**4.2**	**3.3**	**3.3**	**2.1**	**3.1**	**1.0**	**1.3**	**1.1**	**0.6**	**0.5**	**0.7**	**0.4**	**0.4**	**0.8**	**0.5**
Relative Risk (λ)	20	16	16	10	15	5	6	5	3	2	4	2	2	4	2
(95% confidence interval)	(15 to 26)	(13 to 20)	(12 to 22)	(7 to 14)	(13 to 17)	(2 to 9)	(2 to 13)	(3 to 8)	(1 to 4)	(0.9 to 4)	(3 to 5)	(1.2 to 3)	(0.2 to 6)	(1.0 to 8)	(1.5 to 3)

NOTES: Prevalence of oral clefts in the background population born in Denmark between 1952 and 2005: (7,619)/(3,703,337) = 0.21%

Confidence intervals are computed from $C*(\sqrt{a} \pm \frac{1}{2}Z_{\alpha/2})^2/n$, where a = no. of affected relatives of type R, n=total no. of relatives of type R, α=0.05 and C=100 for the confidence interval of the risk in percentage and C=(1/prevalence in the background population) for the confidence interval of the relative risk

iCL: isolated cleft lip; iCLP: isolated cleft lip with cleft palate; iCP: isolated cleft palate

* Number of relatives affected by an oral cleft (including syndromic oral cleft and oral cleft with associated anomalies)

† For computation of the recurrence risks for siblings, twins are excluded from both groups of the probands and their siblings; the numbers of iCL, iCLP and iCP probands are respectively 2,055, 2,487 and 2,044.

‡ Grandparents of probands are born between 1936 and 2005

When the recurrence risk for siblings was stratified by severity, e.g. from bilateral CLP to bilateral CLP, it was seen that the most severe cleft type for both CL and CLP tends to recur, table 2. The only exception from this pattern was for CP where the moderate severity (from soft cleft palate to soft cleft palate) had the highest recurrence risk (3.9% (95% confidence interval 2.5% to 5.6%)), although not statistically different from the severest form.

Table 2: Recurrence Risk for siblings of having the same phenotype of cleft as the probands according to laterality or severity of clefting (Denmark, 1952-2005)

Phenotype	Sub-phenotype	Total number of siblings	Recurrence for siblings		
			Number	Risk (%) (95% confidence interval)	p (heterogeneity)*
iCL probands	Unilateral	1,977	27	**1.4** (0.9 to 1.9)	0.50
	Bilateral	205	4	**2.0** (0.5 to 4.3)	
iCLP probands	Unilateral	1,963	49	**2.5** (1.8 to 3.2)	0.004
	Bilateral	854	39	**4.6** (3.2 to 6.1)	
iCP probands	Sub-mucous CP	659	18	**2.7** (1.6 to 4.1)	
	Soft CP	622	24	**3.9** (2.5 to 5.6)	0.75
	Soft-Hard CP	999	26	**2.6** (1.7 to 3.7)	

NOTES: 11%, 5% and 5% of siblings for respectively the iCL, the iCLP and the iCP probands are not included in these numbers because of unknown sub-phenotype of the probands
Twins are excluded from both groups of the probands and their siblings.
iCL: isolated cleft lip; iCLP: isolated cleft lip with cleft palate; iCP: isolated cleft palate
* Pearson Chi-squared test

When looking at the recurrence risk within each subtype of OC for siblings, e.g. from CLP to CLP, a consistent pattern of recurrence specificity was shown. The highest recurrence risk was found to the same subtype within all three subtypes (table 3). For the two known distinctly different defects CL(P) and CP, we found a crossover risk that was significantly lower than the recurrence risk within the type, but slightly higher than the risk in the background population (e.g. for CP to CLP 0.2% (95% confidence interval 0.0 to 0.4%).

Table 3: Specificity of the recurrence risks for siblings (Denmark, 1952-2005)

Phenotype	Total number of siblings	Recurrence for siblings					
		Number			Risk(%) (95% confidence interval)		
		iCL	iCLP	iCP	iCL	iCLP	iCP
iCL probands	2,442	35	24	0	**1.4** (1.0 to 1.9)	**1.0** (0.6 to 1.4)	-
iCLP probands	2,954	22	87	4	**0.7** (0.5 to 1.1)	**2.9** (2.4 to 3.6)	**0.1** (0.0 to 0.3)
iCP probands	2,379	0	4	67	-	**0.2** (0.0 to 0.4)	**2.8** (2.2 to 3.5)

NOTE: Twins are excluded from both groups of the probands and their siblings.
iCL: isolated cleft lip; iCLP: isolated cleft lip with cleft palate; iCP: isolated cleft palate

The recurrence risk for offspring was also stratified by whether the relatives were on the maternal or paternal side of the case. For the CLP that is predominant in males, we found the highest recurrence risk for children when the mother was affected and for the CP with female predominance we found the highest recurrence risk for children when the father was affected (table 4). Within each phenotype the recurrence risks were, however, not statistically significant.

Table 4: Recurrence risk for offspring of having the same phenotype of cleft as the probands by the gender of the affected relative (Denmark, 1952-2005)

Phenotype	Gender	Total number of offspring	Recurrence for offspring		
			Number	Risk (%) (95% confidence interval)	p (heterogeneity)*
iCL probands	Male	865	14	**1.6** (0.9 to 2.6)	0.27
	Female	574	14	**2.4** (1.3 to 3.9)	
iCLP probands	Male	993	19	**1.9** (1.1 to 2.9)	0.11
	Female	598	19	**3.2** (1.9 to 4.8)	
iCP probands	Male	456	15	**3.3** (1.8 to 5.2)	0.35
	Female	755	18	**2.4** (1.4 to 3.6)	

iCL: isolated cleft lip; iCLP: isolated cleft lip with cleft palate; iCP: isolated cleft palate
* Pearson Chi-squared test

The recurrence risk according to family size for full siblings (1,787 siblings, 44 affected) showed the same pattern of increasing risk with an increasing number of children in a family for all sub-phenotypes, e.g. for CLP the recurrence risk increased from 2.0% (95% confidence interval 1.2 to 2.9%) in a family with 2 children to 6.5% (95% confidence interval 1.2 to 16.0%) in a family with 4 children. Though not statistically significant, the direction of the point estimate is clear.

4.2. Twin recurrence (Paper II)

During 1936 to 2004, 207 MZ and DZ twin pairs were born in Denmark, among whom at least one twin was affected with an isolated OC. The index cases were twins discordant for isolated OC who had children (N=117), and their offspring (N=239) born from 1956 to 2005, figure 3.

Figure 3. Twin recurrence of isolated oral cleft, twins born from 1936 to 2004. CL=Cleft Lip, CLP=Cleft Lip and Palate, CP=Cleft Palate, DZ=dizygotic, MZ=monozygotic, UZ=unknown zygosity.
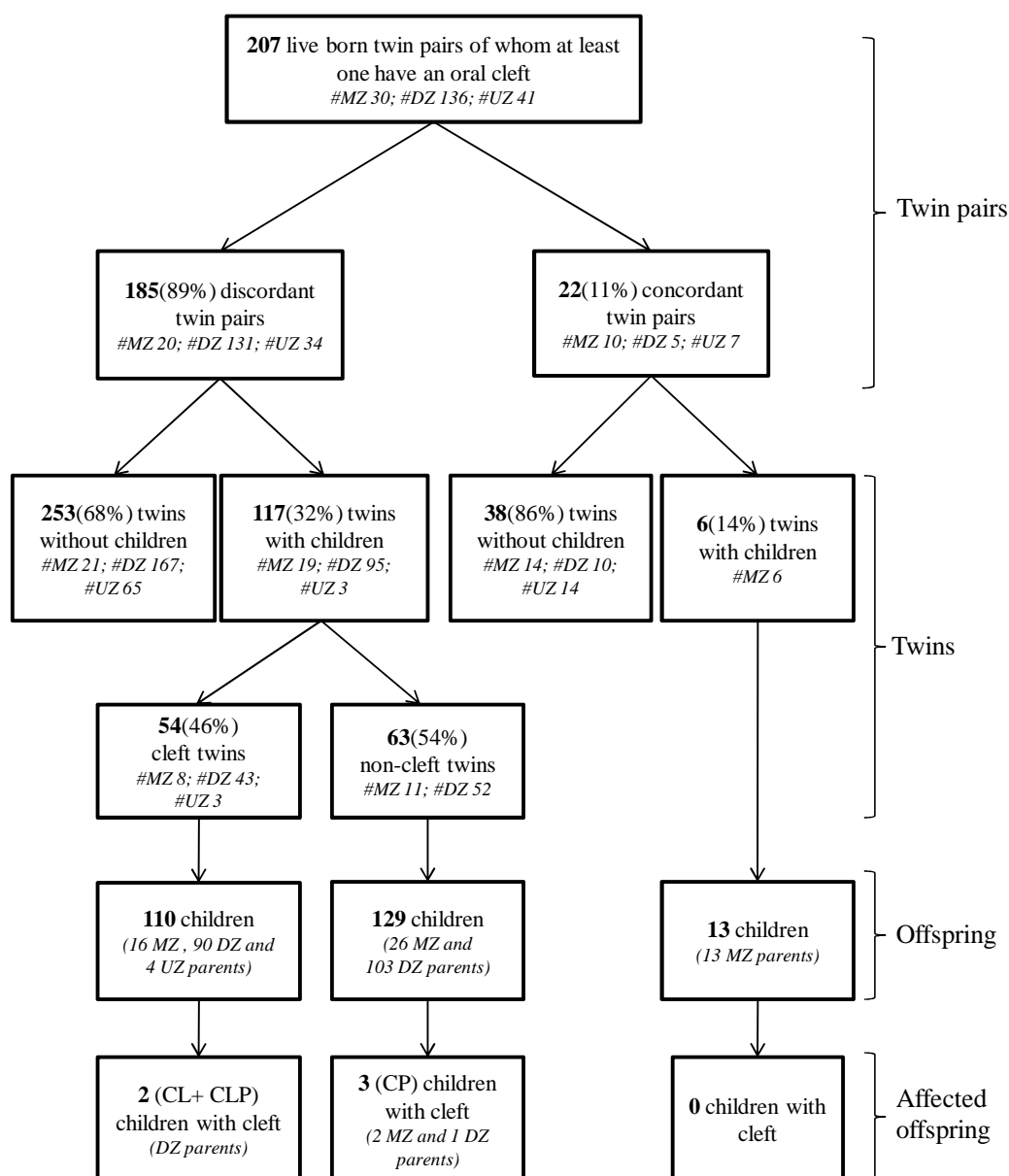
Among the 110 children of the 54 OC twins, two (1.8%) children had OC corresponding to a significantly increased RR on 10 (95% CI 1.2 to 35) when compared to the frequency in the background population (table 5). Among the 129 children of the 63 unaffected twins, three (2.3%) children were affected, corresponding to a significantly increased RR on 13 (95% CI 2.6 to 36) when compared to the background prevalence. Both estimates were in the same order of magnitude as the RR on 19 (95% CI 17 to 22) for the recurrence risk in the general population compared to the background prevalence.

Table 5: Recurrence and relative risk of isolated oral cleft, Denmark 1956 - 2005

Designation of Relationship	Number Affected (n)	Total (N)	Recurrence Risk (%) [95% confidence interval]	Relative Risk*, [95% confidence interval]
Background population prevalence	6,194	3,394,923	0.18	Reference
Offspring of affected parents (background population)	234	6,642	**3.5 [3.1 ; 4.0]**	**19 [17 ; 22]**
Offspring of affected discordant twins	2	110	**1.8 [0.22 ; 6.4]**	**10 [1.2 ; 35]**
Offspring of non-affected discordant twins	3	129	**2.3 [0.48 ; 6.7]**	**13 [2.6 ; 36]**

Significant if p < 0.05, in bold, *Compared to the risk in the background population born in the same time period

The proportion of MZ parents to all offspring of the discordant twins and to the recurrent cases was computed from the numbers displayed in figure 3. Of all the 117 discordant twins with children, 19 (16%) were MZ but two of the five (40%) twins with children who also had an OC were MZ (p = 0.20).

Table 6 displays the OC recurrence risk and the RR for unaffected and affected twins, respectively, stratified for zygosity. The MZ affected twin parents had no affected offspring. The highest recurrence risk was seen for offspring of the MZ unaffected twins and the relative risk was significantly increased (RR = 42; 95% CI 5.3 to 140) when compared to the background population.

Table 6: Twin pairs discordant for isolated oral cleft. Zygosity stratification of recurrence risk and relative risk, Denmark 1956 to 2005

Twin parents status		Recurrence		Recurrence risk (%) [95% confidence interval]	Relative risk*, [95% confidence interval]
Zygosity	Oral Cleft	Number Affected (n)	Total (N)		
Monozygotic	No	2	26	7.7 [0.95 ; 25]	**42 [5.3 ; 140]**
	Yes	0	16	0	0
Dizygotic	No	1	103	0.97 [0.025 ; 5.3]	5.3 [0.17 ; 29]
	Yes	2	90	2.2 [0.27 ; 7.8]	**12 [1.7 ; 43]**

Significant if p<0.05, in bold, *Compared to the risk in the background population born in the same time period

When the OC recurrence risk for the unaffected MZ twins was compared to the OC recurrence risk for the unaffected but DZ twins, the RR was increased (RR = 7.9; 95% CI 0.75 to 85), although not statistically significant. For the DZ twins, the RR for the affected twins was increased (RR = 2.3; 95% CI 0.21 to 25) when compared to the unaffected DZ twins, but also statistically not significant.

The OC twin sample did not have sufficient size to stratify for OC sub-phenotypes.

4.3. Twin prevalence and heritability (Paper III)

Of the 130,710 twins and 4,798,526 singletons born from 1936 to 2004, 207 twins and 7,966 singletons were born with an isolated OC.

In figure 4, the prevalence proportion ratios (PPR) for OC twins relative to singletons are summed up. The prevalence of OC was similar for twins and singletons (15.8 and 16.6 per 10,000, respectively; PPR = 0.95; 95% CI 0.83 to 1.10) for the 1936 to 2004 cohorts. Twins were less likely to have CP compared to singletons (PPR = 0.63; CI 0.53 to 0.76).

Figure 4. Prevalence Ratio for Oral Cleft Twins vs. Singletons
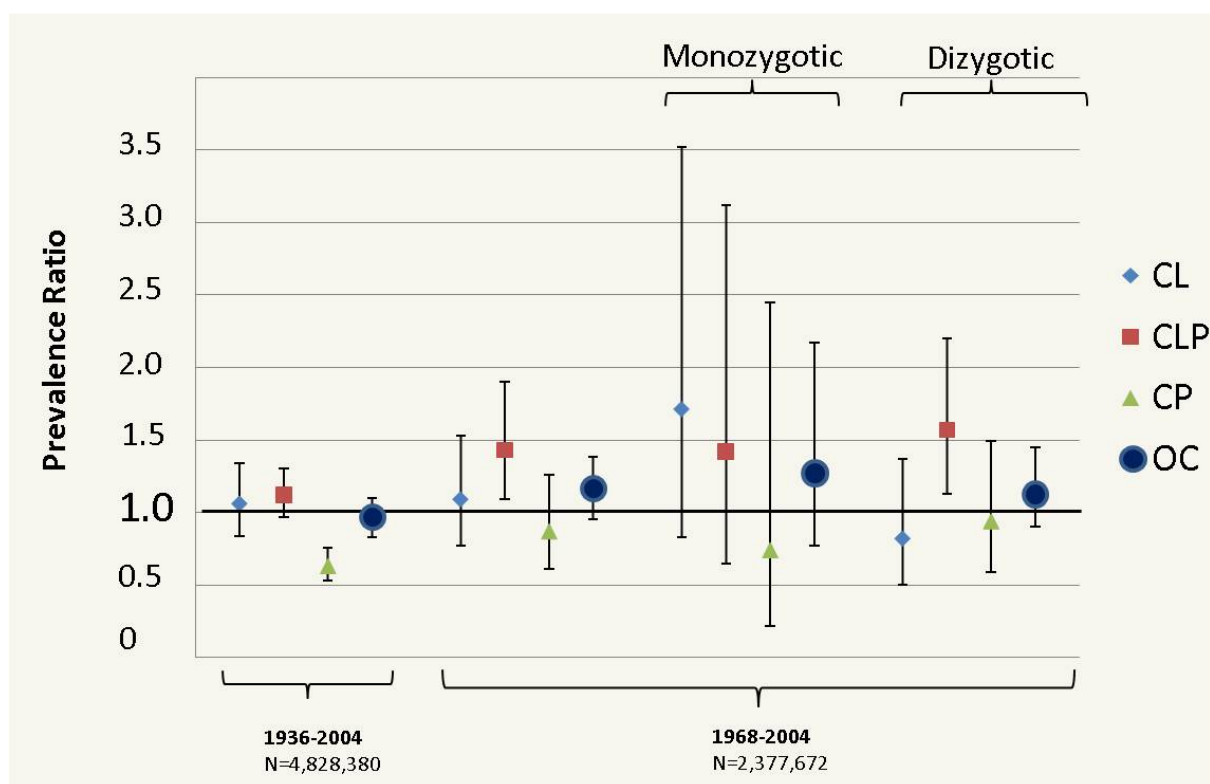
Abbreviations: CL, cleft lip; CLP, cleft lip with cleft palate; CP, cleft palate; OC, all oral clefts

When stratifying into two time periods with a cut-point in 1968 corresponding to the establishment of the CRS, the OC prevalence was lowest (PPR = 0.73; 95% CI 0.73 to 0.93) for the twins before 1968 due to much fewer CPs among the twins for both sexes. From 1968 to 2004, the OC prevalences for twins and singletons were comparable (PPR = 1.15; 95% CI 0.95 to 1.38), but the twins had a significantly higher prevalence of CLP than other phenotypes compared to singletons (PPR = 1.43; 95% CI 1.09 to 1.90).

Comparable PPR's were found for MZ and DZ twins relative to singletons for all of the OC phenotypes with the exception of DZ twins with CLP in whom the prevalence was increased (PPR = 1.57; 95% CI 1.13 to 2.20) due to an increased prevalence for the DZ twin boys (PPR = 1.78; 95% CI 1.23 to 2.59). A similar pattern was seen for twins with UZ (OC PPR = 1.08; 95% CI 0.79 to 1.49).

There was no significant interaction between sex and zygosity. The DTR identified 110,556 of the 130,710 twins (85%) registered in DST in the complete time period, but the ascertainment was nearly complete (99%) from 1968 to 2004.

The C_{Pr} for MZ and DZ twins, recurrence risk for singleton siblings and the background population prevalence are displayed in figure 5/table 7.

Figure 5. Probandwise concordance rates for mono- and dizygotic twins, recurrence risk for singleton siblings, and population prevalence for CL(P) and CP
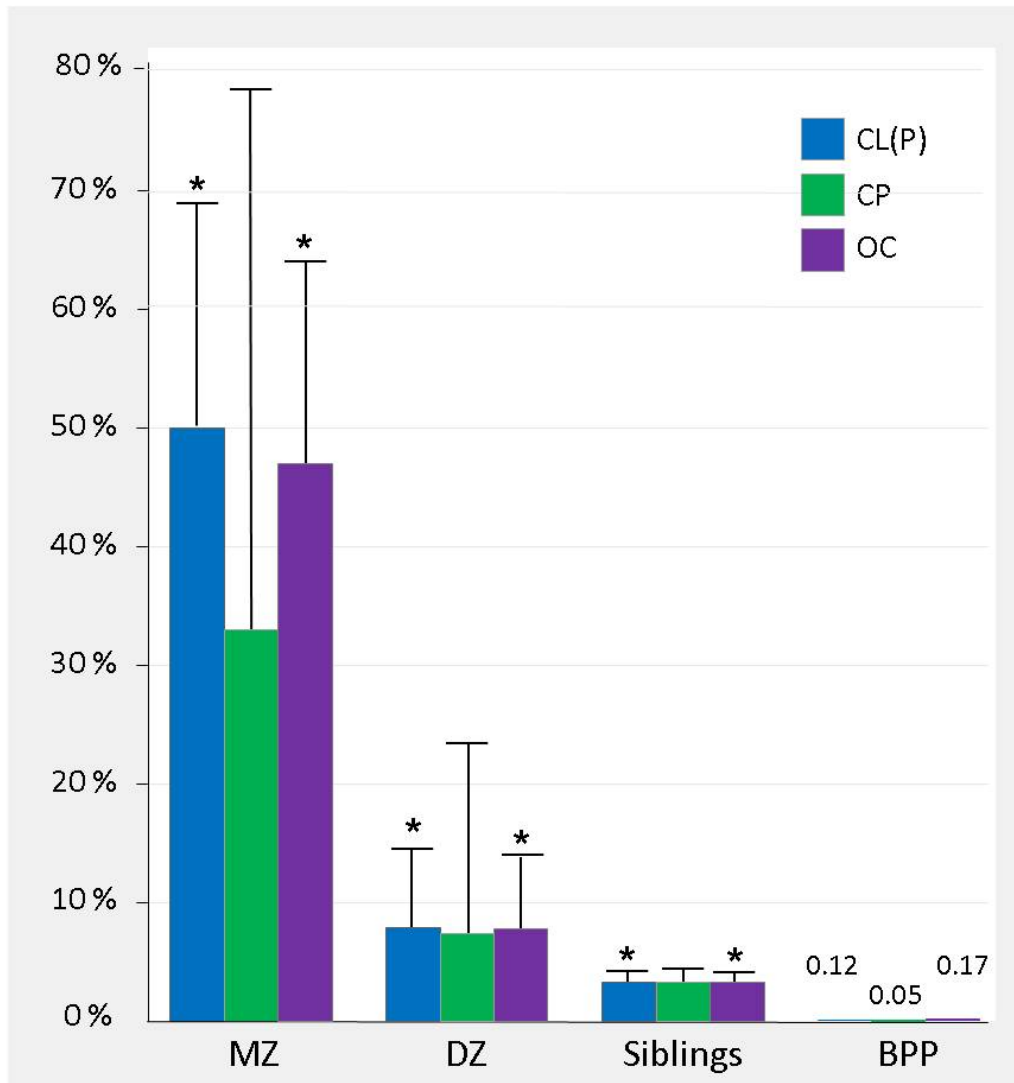
Abbreviations: BPP, Background Population Prevalence; CL(P), Cleft lip with or without cleft palate; CP, cleft palate; DZ, dizygotic; MZ, monozygotic; OC, oral clefts

For CL(P), the C_{Pr} for MZ twins of 50% was significantly higher than the C_{Pr} of 7.9% for the DZ twins. For CP the same pattern with a higher C_{Pr} for MZ twins (33%) compared to DZ twins (7.4%) was found, though not statistically significant. The OC recurrence risk estimates for siblings were derived from the Danish 1952 to 2005 cohorts (paper I). When comparing the C_{Pr} for DZ twins to the recurrence risk for singleton siblings, the C_{Pr} was highest for both phenotypes but only statistically significant for CL(P). When stratified for sex and the three sub-phenotypes, the confidence intervals were wide but the pattern was consistent with a C_{Pr} range for MZ twins from 33% to 67%, for DZ twins from 6% to 12% and for UZ twins from 13% to 33%.

Table 7. Probandwise concordance rates for twins and recurrence risk for siblings for isolated OC, Denmark 1936-2004 (N=185 twin pairs/7,654 sib pairs)

Phenotype	MZ		DZ all		DZ same sex		UZ		Siblings		p value for comparison^	
Concordance	N	C_{Pr},% (95% CI^)	N	C_{Pr},% (95% CI^)	N	C_{Pr},% (95% CI^)	N	C_{Pr},% (95% CI^)	N	Recurrence risk*, % (95% CI^)	MZ vs. DZ all$	DZ all vs. siblings
Cleft lip with or without cleft palate												
Concordant pairs	8	50 (32-68)	4	7.9 (3.5-15)	2	7.7 (2.1-19)	5	31 (16-50)	86	3.2 (2.7-3.7)	<0.0001	0.02
Discordant pairs	16		93		48		22		5224			
Cleft palate												
Concordant pairs	1	33 (4.3-78)	1	7.4 (0.91-24)	0	0	0	0	36	3.0 (2.3-3.8)	0.15	0.20
Discordant pairs	4		25		17		6		2308			
Oral Cleft												
Concordant pairs	9	47 (31-64)	5	7.8 (3.8-14)	2	5.6 (1.6-14)	5	26 (13-43)	122	3.1 (2.8-3.5)	<0.0001	<0.01
Discordant pairs	20		118		67		28		7532			

*Recurrence risk from 1952 to 2005

^Exact methods for 95% confidence intervals (CI) and significance testing, significance level < 0.05

$Under the assumption of equal prevalence for MZ and DZ twins

In table 8, the tetrachoric correlations and variance component analyses for CL(P) and CP for all twin pairs from the 1936 to 2004 cohorts are displayed. The highest tetrachoric correlation was found for MZ twins for both phenotypes when compared to DZ twins, statistically significant for CL(P). For both CL(P) and CP the best fitting model was the AE model (lowest AIC). For CL(P) and CP the heritability estimates (a^2) were very similar: 91% and 90% respectively, and the unique environmental factor (e^2) was small; 9% and 10% for CL(P) and CP, respectively. The estimates did not take difference in sex into account due to lack of sample size.

Table 8. Tetrachoric correlations and variance component analyses for Danish twins with isolated Oral Cleft, 1936-2004 (N=185)

N	Cleft lip with or without cleft palate			Cleft palate		
MZ pairs[*]	8/16			1/4		
DZ pairs[*]	4/93			1/25		

Zygosity	r (95% CI)	p Value		r (95% CI)	p Value	
MZ	0.91 (0.79 - 0.97)	<0.001		0.88 (0.47 - 0.99)	0.20	
DZ	0.55 (0.35 - 0.70)			0.60 (0.29 - 0.83)		

					Model Fit Statistics				
	vs.	x^2	d.f.	AIC	p Value	x^2	d.f.	AIC	p Value
ACE	SAT	0.78	3	-5.23	0.86	0.12	3	-5.88	0.99
ADE	SAT	1.76	3	-4.24	0.62	0.94	3	-5.07	0.82
AE	*ACE*	*0.99*	*1*	*-6.24*	*0.32*	*0.82*	*1*	*-7.07*	*0.37*
CE	ACE	13.60	1	6.37	<0.001	1.64	1	-6.24	0.20
E	AE	107.97	1	99.73	<0.001	20.24	1	11.36	<0.001

Heritability estimates (95%CI)	a^2	e^2	a^2	e^2
	0.91 (0.82 - 0.97)	0.09 (0.03 - 0.18)	0.90 (0.60 - 0.99)	0.10 (0.01 - 0.40)

Abbreviations: A, additive genetic factors; AIC, Akaike's Information Criterion; a^2 and e^2, standardized parameter estimates (95% CI); C, common environmental factors; CI, confidence interval; d.f., degrees of freedom; DZ, dizygotic; D, dominant genetic factors; E, non-shared environmental factors; MZ, monozygotic; SAT, saturated model; r, tetrachoric correlations

[*]Complete pairs (i.e. both twins have a score)/broken pairs (i.e. only one twin has score)

Note: Best fitting model in *italic*

5. Discussion

5.1.   Paper I

The recurrence risk estimates on first, second and third degree relatives were in good agreement with the recurrence risk estimates on the first report on a smaller subset of the Danish population for the isolated OCs born between 1952 and 1987[6,7]. The precision of the estimates has been increased markedly and has benefitted from an increase in sample size by a factor ten for numbers of phenotyped relatives. For the first time it was possible to present reliable estimates for first cousins (and other third degree relatives) of individuals affected with a CL, CLP, or CP.

For first degree relatives a higher recurrence risk among offspring and siblings compared to parents was observed along with a tendency to repeat the same cleft type in the recurrence, a strong effect on recurrence according to severity, the highest recurrence risk in the least frequently affected sex, and a steep drop-off in the recurrence risk from first to second degree relatives and from second to third degree relatives. A tendency towards an increasing recurrence risk for full siblings with an increased number of sibs was observed, but the results did not reach formal significance. These results support the multifactorial threshold model of inheritance and cannot support a new suggestion of a shift away from the use of the multifactorial threshold model of inheritance explaining the joint action of genes and the environment on the OC risk[45]. The Norwegian study used a different severity classification than the one traditionally used. When re-classified in order to be comparable to the severity classification in this study, a consistent pattern of repeating the most severe cleft type for all cleft types including CP was found. This model has been in use since the 1960s[31-33] and despite several challenges[35,36,45] it still appears to be the best model to explain the etiology of oral clefting[6,7,37-43].

5.2.   Paper II

The OC recurrence risk for offspring of twins discordant for OC was similar regardless of whether the twin was affected, i.e. the unaffected twin's risk for an affected offspring was not significantly different from that of the affected twin. In addition, the recurrence risk for offspring of both the affected and unaffected twin from a twin pair discordant for OC was significantly increased compared to the background population frequency.

Since MZ twins share 100% of their genes as opposed to 50% as for DZ twins and since the etiology to OC is mainly genetic, the discordant MZ twins should both be carrying susceptibility genes for OC. The affected twin could have an additional novel mutation acquired after the division of the zygote. That mutation may have pushed this twin over the

threshold of developing OC or a difference in expressivity may have caused the discordance. When reproducing, both twins would then pass on susceptibility genes, but since the one twin exceeded the threshold, it could be expected that its risk would be the highest[28;29]. The highest risk was found for offspring of the unaffected twins relative to the background population risk, although the confidence intervals were wide. This indicates that offspring of the unaffected co-twin of a discordant pair have an increased liability to OC and likely a similar liability as offspring of affected twins. Because of the strong genetic component in the OC etiology[8;172], it is plausible that the increased liability for the unaffected twins may be due to them carrying susceptibility genes for OC as the affected twins were most likely to have done. Another explanation could be that both twins had been exposed to an environmental factor while in the womb, which later increased the risk for their offspring when they reproduced.

The similar OC recurrence risks for twin offspring and offspring of affected parents from the background population indicates that the mechanism of clefting is the same whether the parent was a twin or not. This hypothesis was tested using a stronger design in paper III.

The overall recurrence risk was higher among offspring of the unaffected twins despite inclusion of the DZ twins. When the results were stratified by zygosity, the highest risk was found for offspring of the unaffected MZ twins and this risk was eight times higher than the risk for offspring of the unaffected DZ twins. Along with a tendency towards an increased proportion of MZ twin parent to recurrent cases compared to the proportion of MZ twin parents to all offspring, it was most likely the recurrence risk for offspring of MZ twins that drove the overall recurrence risk estimate for unaffected twins. These results add further evidence for a genetic etiology to oral clefting, but they do not rule out yet unmeasured environmental factors.

In 2002 Kondo et al reported on a pair of MZ twins discordant for Van der Woude syndrome in which oral clefting is a major manifestation. They sequenced a large section on chromosome 1 that had been identified through genetic linkage studies and found a mutation in the *IRF6* gene that is now known to be the cause of Van der Woude syndrome. This strategy had been used for another Mendelian disorder[65] and was subsequently applied to isolated OC. These studies have used MZ twins discordant for isolated OC, but have failed to identify differences in genes of importance for oral clefting[63;80], differences in copy number variations[63;80] or in X-chromosome inactivation patterns[62]. The major difference between the two disorders is that Van der Woude syndrome is a monogenic disease where isolated OC is a multifactorial trait. The results presented can provide an additional explanation as to why this otherwise reasonable twin approach continues to fail. The unaffected twins were carrying a liability for oral clefting e.g. susceptibility genes for oral clefting. They had, however, not reached the threshold for developing an overt cleft, either by chance, due to low penetrance, or due to variable gene expression as seen for *IRF6*. Mutations in *IRF6* can result in tooth agenesis for some individuals, isolated clefts or syndromic forms of clefts for other individuals[92].

Moreover, the twins could have had a microform of OC such as a defect in the orbicularis oris muscle hence not routinely registered. As mentioned, several studies have shown that microforms of OC are seen more frequently among unaffected relatives, and a large multicenter study is currently exploring this finding in greater detail[13-18]. If the control group of unaffected individuals is contaminated with affected individuals (OC microforms), the ability to detect an association is weakened. Future studies including the microforms of OC can both increase the study population and diminish the risk of missing a true association between genes and OC occurrence.

5.3.   Paper III

No excess risk of OC for twins compared to singletons could be found using a twin sample three times the size of the previously exploited Danish twin sample. For the youngest cohorts, the OC risk for twins was slightly increased, but the risk could not be distinguished from the singleton risk. This upwards nudge in the OC prevalence for twins compared to singletons for the youngest cohorts was likely due to a decrease in the infant mortality over time for twins in general and OC twins in particular. Nor could an excess risk of OC be demonstrated for the MZ twins when compared to singletons. The highest concordance was found for MZ twins, along with very high heritability estimates which provide further support for a substantial genetic contribution to the OC etiology. However, a new indication of DZ twins having an excess risk of OC compared to singleton siblings justifies the continuing search for environmental factors of importance for the OC etiology.

This study population is an expansion of the data exploited by Shields et al.[168] in 1979 and Christensen and Fogh-Andersen[8;9] in 1993. The OC occurrence among twins and singletons has also been studied in other populations, and in the largest studies C_{Pr} and heritability were estimated. Some studies were too small to draw anything else but a hypothesis[48;51]. Others were large but susceptible to either ascertainment bias[53;173], inclusion of syndromic forms of OC[9;57;58;173], or without stratification for zygosity[49;50;56]. The majority of all these studies found no difference in the OC prevalence for twins relative to singletons and these studies were based on a total number of twins more than twice the size of the twin sample used in the studies suggesting a difference[8;9;50;52;54;56;58-61;168;174]. The largest of the previous studies also estimated pairwise or probandwise concordance rates [8-10;58;61;168;174], only one recent study estimated heritability (CL(P): a^2=0.73; standard error 0.42 and a^2=0.66; standard error 0.39, male and female respectively)[8].

The size and the quality of the Danish twin sample overcame most of the challenges mentioned and provided valid estimates of OC occurrence for twins and singletons, C_{Pr} and heritability. The decreased risk for twins found by Shields et al. in a subset of the Danish population can, together with the results presented in this study, be ascribed to survival bias. The excess OC

concordance for MZ twins compared to DZ twins supports the evidence of a large genetic component to the etiology of OC and the demonstration of a more than fourfold increased C_{Pr} for MZ twins relative to DZ twins agrees with the finding that several loci have an effect on the OC etiology[6;7;44]. Nevertheless, the less than 100% phenotypic concordance indicates that environmental factors could be of importance since the genomic sequence alone cannot explain the disease susceptibility. The effect of the environment is supported by the excess OC risk for DZ twins relative to singleton siblings, which could be demonstrated for the first time in this study. This difference justifies the continued search for environmental factors of importance for the cleft etiology to help prevent OC in the future. However, environmental effects may not sufficiently explain the MZ twin discordance which could also result from genetic, cytogenetic or epigenetic anomalies in the affected twin, and not the other[62;63;80].

A measure of the magnitude of the genetic and environmental effect was provided by use of a variance component analysis. More than 90% of the variation in liability to OC could be explained by genetic effects for both CL(P) and CP. The best fitted model in the variance component analysis was the AE model suggesting that the proportion of variance in the OC occurrence is solely due to additive genetic factors (A) and unique (non-shared) environment (E).

5.4.   Strengths and weaknesses

The reliability of the studies depends upon the precision and validity of the estimates. The precision is related to sample size (random error) and the validity is related to selection bias, information bias and confounding (systematic errors) [175].

5.4.1.   Power and significance

The four registers used for this thesis are all national and population-based. The studies benefitted from the longstanding ascertainments in the DFCD and the DTR, and for these registries the ascertainment has been considered complete since the establishment of the CRS in 1968. Both registries used several valid data sources for the ascertainment which has improved markedly over time resulting in large data samples. The recurrence risk estimates benefitted from an increase in sample size by a factor ten for numbers of phenotyped relatives[6;7]. The recurrence risk for the unaffected twins in the OC discordant twin pairs was without precedent and when estimating the OC prevalence for twins, the sample had increased by a factor three when compared to previous estimates on the Danish 1970-1990 cohorts(207 twins vs. the previous 65)[8]. Moreover, when restricting the analyses to the youngest cohorts from 1968 to 2004, the study benefitted from complete ascertainment for all individuals, available zygosity for 80%, and still a twin sample twice the size of the previous Danish sample (136 twins vs. the previous 65)[8;9].

With the large samples available, very precise estimates of recurrence risk for first, second, and third degree relatives could be provided. When estimating the recurrence risk for twins discordant for OC, the power for the study was significantly weakened due to the relative rarity of co-occurring twinning and clefting. From 1936 to 2004, the average twin frequency was 1.3%. With an OC prevalence of 0.17% for the same period, the probability of twinning to co-occur was about one in 45,000 individuals. In paper III many subgroup analyses were made resulting in thin strata and reduced power for the estimates. Another downside of the many subgroup analyses is the chance findings which cannot be ruled out at the 5% significance level chosen. Moreover, caution should be taken when making conclusions on differences found in subgroup analyses when no overall difference in the OC prevalence between twins and singletons were found.

Similar data from other Nordic countries could provide an additional source to obtain estimates stratified for zygosity, cleft sub-phenotype and gender in the latter two studies.

5.4.2.   Bias

5.4.2.1.   Selection bias

Linkage between the DFCD and the DTR is dependent on an available PIN for each individual. For the complete OC cohort, 9% of the individuals were lacking a PIN and for the 1952 to 2005 cohort the number was 6%. Those individuals were either not alive in 1968 or could not be uniquely identified from the original surgical records made by Dr. Poul Fogh-Andersen. Since they could not be linked to other relatives or registries they had to be excluded from all analyses. In order to visualize whether this possible selection bias had resulted in an underestimation of the recurrence risk estimates provided, similar analyses were performed in paper III for the 1968 to 2005 birth cohorts in whom fewer PINs were missing (3%). The point estimates remained virtually unchanged.

A decrease in neonatal mortality rates over time might have introduced a selection bias into the DFCD for the earliest cohorts. Before 1954, where midwifes in Denmark became obliged to report any OC identified at birth to the National Institute of Defect of Speech, the OC individuals had to survive until the age of 2 months to be evaluated for surgery and thereby be included in the DFCD[165]. The individuals with a CP were most susceptible to this survival/selection bias because they often entered the database later in life, since they were evaluated for surgery when they were two years old[165]. Moreover, the CP individuals with an associated syndrome or other anomalies may have had an even higher infant mortality since CP individuals were twice as likely to have an associated syndrome or other anomalies as the CL/CLPs[1]. Individuals with the mildest CP form, which could easily be overlooked, were also prone to selection bias since they were not evaluated for or in need of surgery, and

hence not included in the database. For the OC twins, the survival/selection bias might have been magnified since at least one third of all the twins were born preterm with an accompanying higher infant mortality. After 1954, all OCs also registered later in a child's life were reported to the institutes which, along with the improvement of the neonatal care from the 1960s, has improved the ascertainment markedly. This survival/selection bias was not likely to influence the recurrence risks for first, second, and third degree relatives since the cohorts were restricted to 1952 to 2005 and the even more restricted analysis from 1968 and onwards did not change the estimates. Since also the earliest cohorts of twins were included in paper II, the estimates of the recurrence risk for the OC discordant twins might have overestimated the recurrence since more affected individuals would have been ascertained among the offspring compared to the parents (the twins), but the RR estimates are unaffected. In paper III the underascertainment of the CPs becomes evident since markedly fewer CP twins are ascertained compared to singletons from 1936 to 1967, and it is not plausible that the smaller number of CPs was due to a difference in the diagnosing of CP or OC in general for twins relative to singletons.

The total twin population when drawn from the DTR also suffers from survival bias since the twins in the oldest cohorts had to survive until the age of 6 to be included in the Registry[166]. In paper III this underascertainment of twins in the DTR relative to the DST is visualized since the ascertainment for the complete time period was 85%, but nearly complete (99%) from 1968 to 2004 .

Another factor that may have biased the recurrence risks towards an underestimation is the fact that only legally identifiable parental links are used in the CRS so in the case of adoption or non-paternity, the children cannot be identified. In the DFCD, however, only individuals born in Denmark are included. According to the DST adoptive children comprise a maximum of 1.5% of all the birth cohorts from 1952 to 2005, and about 90% of them were born outside of Denmark and hence excluded from the DFCD[169].

Since the initiation of the update of the DFCD from 1988 to 2005, the ascertainment and classification of associated anomalies/syndromes have been enhanced considerably[1]. For the earliest cohorts it is likely that some degree of misclassification of individuals with undiagnosed associated anomalies/syndromes as individuals with isolated OCs has occurred. However, this only concerns the milder forms of associated anomalies/syndromes since the most severe cases were ascertained in connection with surgery. Since the misclassification entails unintentional inclusion of nonsyndromic forms of OC this bias is rendered a selection bias. An effect of this bias might be the increased crossover risk between clefts involving the lip and those involving the palate only when estimating the recurrence specificity in paper I. It

could, however, also be explained by the chance occurrence of syndromic clefts (included in the study as nonsyndromic) or by genes like *MSX1* and *IRF6* where both types of clefts may appear in the same family and with no additional phenotypic traits resulting in its being assigned to a syndrome category. This possible misclassification of the syndromic OCs as non-syndromic might have resulted in a slight overestimation of the recurrence risk estimates since the inheritance is different and likely higher among the syndromic OCs. Another effect of this misclassification is found in paper II. The recurrences were of similar type (CL/P or CP) except in one family which could represent an undiagnosed case (born in 1946) of Van der Woude syndrome where both CL/P and CP occur. If this one family was excluded, the same overall results were obtained: the recurrence risk for offspring of unaffected twins was 1.6% (95% CI 0.19 % to 5.6 %), not differing from the recurrence risk among affected twins of 1.8% (p = 1.0), and a significantly increased relative risk (RR = 8.7; 95% CI 1.1 to 31) when compared to the background prevalence.

5.4.2.2. Information bias

It has previously been shown that the zygosity determination in the DTR has a high degree of validity[167;176]. However, the use of questionnaires regarding physical resemblance might not be the best method in a study of facial malformation. From studies on two subsets of the Danish OC twin populations from 1941-1969[168] and 1970-1990[8;9] it was evident that the method resulted in less than 5% misclassification of MZ twins as DZ twins. Both studies used blood, serum, and enzyme determinants to verify the information obtained in the questionnaire. When studying recurrence risk for OC discordant twins the bias was differential, but the RRs were unaffected. When estimating the OC prevalence compared to singletons no bias was introduced. In paper III, the OC C_{Pr} for DZ same sex twins was lower than the C_{Pr} for all DZ twins which could indicate such bias (table 7). For the CL(P)s, however, no such difference could be found and since the CL(P) individuals would be the most prone to misclassification due to the major facial asymmetry, any information bias introduced on this behalf was likely to be minimal. This assumption was also supported by the similar MZ:DZ twin proportion for the cleft twin population and the total twin population and comparable proportions of MZ and DZ twins among the CL, CLP or CP individuals.

The DFCD includes overt OCs but for the bifid uvula the ascertainment was insufficient and other microforms like dental anomalies were not registered. With the increasing understanding and acceptance of such microforms it becomes evident that using relatives of an OC individual as a control might not always be the best choice. The control group then contains individuals carrying the same gene variants of importance for oral clefting as do the OC affected individuals. In association studies

using overt OC discordant MZ twins, such misclassification might result in a type II error, missing a true association. In paper II, this misclassification may have influenced the recurrence risks for offspring of the affected and the unaffected twins. Since the misclassification is differential, the effect of it cannot be predicted for the recurrence risks but the RRs should be unaffected. This study visualized the concerns about this bias which has been expressed in studies using discordant MZ twins in genetic association studies. This type of studies might not prove useful until also the microforms are registered.

5.4.2.3. Confounding

The recurrence risk estimates could be overestimated if both parents were affected by an OC. Very few affected spouses were observed, 1.5 per 1,000, which was a little less than the population frequency; hence it is unlikely to influence the results of familial recurrence risk patterns.

The Danish population is in general known to be both homogeneous (over 93% of current births in Denmark have grandparents also born in Denmark) and to have a low incidence of consanguinity among ethnic Danes[177] which enhance a study's power to detect genes/loci contributing to risk. With the increasing immigration to Denmark, both the ethnic admixture and consanguinity might become an issue that should be taken into account in future studies. Since the multifactorial threshold model best describes the inheritance of OC, consanguinity would slightly increase the recurrence risk[178]. The effect of ethnic admixture will be dependent upon where the immigrants come from[24].

The recurrence risk for siblings might be biased if parents had fewer children than expected after having a first child born with oral cleft. If that is the case, the risk for all siblings would be underestimated and different from the risk for the later-born siblings. Both risks were computed and the results support no such assumption.

6. Conclusion and perspectives

6.1. Conclusion

In paper I exact OC recurrence risk estimates for first cousins were provided for the first time. The recurrence risk was increased when compared to the background population, but to a lesser extent than the recurrence risk for second degree relatives of an OC individual, and this again was lower than for first degree relatives – approximately 0.2% : 0.5% : 0.7% : 3%, respectively. This pattern of recurrence along with recurrence risks stratified for severity, specificity, parent of origin effect, and family size for first degree relatives support the multifactorial threshold model of inheritance which, in 2008, was challenged by a Norwegian study. The results will help improve the counseling of family members of OC individuals.

With access to an OC twin sample three times the size of the previously exploited Danish sample (1970-1990), it was possible to further explore recurrence risk patterns, e.g. for twins discordant for oral clefting. The OC recurrence risk for offspring of twins discordant for oral clefting was compared and both risks were compared to the risk in the background population. The OC recurrence risk was the same whether the twin was affected or not, and it was increased compared to the background population. The similar recurrence for offspring of the affected and the unaffected twins indicated that both twins were carrying a genetic vulnerability for oral clefting. The results, supplementing the recurrence risks provided for first, second, and third degree relatives, can be used in the rare genetic counseling situation of twin pairs discordant for oral clefting. Furthermore, these results support the major role of genes in OC etiology and point to the need for a better understanding of OC sub-phenotypes.

Finally, the large OC twin sample made it possible to make a more powerful test of whether twinning has an effect on the risk of isolated OC by comparing the OC occurrence among twins and singletons. A precise estimate of the relative contribution of genes and the environment to the OC etiology was provided by use of C_{Pr}, tetrachoric correlations, and heritability estimates. No excess risk of OC for either all twins or MZ twins compared to singletons could be found. The highest degree of concordance was seen for the MZ twins (the C_{Pr} was approximately 50% for MZ twins and 8% for DZ twins (p=0.01)) along with very high heritability estimates for both CL(P) and CP (>90%). Moreover, for the first time it was indicated that DZ twins have an excess risk of OC compared to singleton siblings, which provides additional justification for the continuing search also for environmental factors of importance for the OC etiology.

6.2.   Public health implications

The public health implications of this thesis are mostly related to genetic counseling of family members to OC affected individuals in Denmark. With the highly reliable recurrence risk estimates provided, even first cousins can now be advised about the increased risk for their children. Even in the rare situation where an unaffected co-twin to an OC affected twin seeks advice, genetic counseling can be provided. The unaffected twins should be informed that their risk is the same as the risk of their co-twins even though they are not affected themselves. The pattern of inheritance can also be useful in the genetic counseling situation. For example, parents to an already affected child can be informed that, in their next child if this child is affected by an OC, the cleft will most likely be of the same type and severity as the one their firstborn child was born with. Since these results are national and population-based, they apply to the Danish population, but might not be generalizable to other populations due to differences in ethnicity, both within and between the populations. This thesis has pointed to the need to identify other sub-phenotypes of OC in order to broaden the OC phenotype. If the sub-phenotypes can be ascertained, improved recurrence risk estimates can be provided and the chance of identifying genes of importance for OC using the discordant MZ twin design will be increased. All studies support a major genetic contribution to the OC etiology, and since no excess risk of OC could be shown for twins relative to singletons, these results are likely to apply also to the general population of, mainly singleton, affected individuals.

6.3.   Future research

The DFCD is among the best resources for epidemiological research concerning OC in the world as stated by Mossey, 2007, accurate epidemiologic information and data underpin other research and clinical trials by (1) assessing the burden of OC in order to plan public health resources and strategies, (2) assessing causes of OC, and (3) providing a scientific basis for evaluating the scope for intervention strategies[24]. In 2006 the International Database on Craniofacial Anomalies (ICDFA) was therefore designed. Danish OC information collected through EUROCAT (a European network for surveillance of congenital anomalies) on Funen is shared with ICDFA. Data collected on Funen accounts for about 10% of the total Danish population and is considered to be representative for the rest of the country. The present thesis emphasized that, despite the OC etiology being mainly genetic, the search for environmental factors are still justified in order to establish possible prevention strategies. With the access to epidemiological data from the DFCD, new surveys can be established in order to collect additional case and control information on putative risk factors, both genetic and environmental. However, the DFCD data and other OC survey data have recently been linked to DST's register, which allows for immediate analysis of the effect of various risk factors since the information has already been collected. Not only will it be possible to continue the search for factors of importance to the OC etiology like drugs administered to pregnant women and

maternal illness, but it also enables studies of the life course of children born with OC as well as their first degree relatives. Some long term consequences using Danish data have been described already, like the increased overall mortality[3], the increased risk of psychiatric diseases[4], and an indication of an increased association with breast cancer[5]. The combination of the DFCD with DST provides unprecedented opportunities to study both rare and common long term consequences. Studies of

- academic performance,
- the socioeconomic trajectory,
- health (measured by hospitalization and out-patient services (general practitioners, prescription medicine etc.)),
- cause-specific mortality,
- associated morbidities,
- and associated anomalies

for OC cases compared to the background population as well as siblings to OC cases (to control for familial factors) can be performed. If the results from these studies of long term outcomes can be tied with genetics then high risk populations, that will benefit for more intensive or targeted screening to reduce adult onset disease, can be identified.

Future studies would benefit greatly from including the microforms of OC since the specificity and the power of genetic studies will be improved when using unaffected relatives as controls. Furthermore, sub-clinical phenotyping shows great promise in changing the paradigm of phenotyping within cleft families, leading to important translational opportunities for cleft care and clinical genetics where the recurrence risk estimates could be refined and personalized[160]. In order to include these sub-phenotypes, the type and frequency must be determined. As mentioned, evidence from several studies have shown that alterations in skull dimensions, dental anomalies, and disruption of the orbicularis oris muscle etc. can be classified as such a sub-phenotype[13-18;179]. A large multicenter study, including Danish participants, is currently exploring these findings into greater detail. Eligible candidates for the study are the twins discordant for OC from paper II and their families, and large multiplex families.

7. Summary in English

Oral clefts (OC) are among the most frequent congenital malformations and they have significant adverse health, social, and economic ramifications. Non-syndromic oral clefts can be defined as complex traits since they exhibit no classical Mendelian inheritance, but show strong familial aggregation and have a substantial genetic component. About 25% of the isolated OCs can be attributed to known gene variants and approximately 5% to smoking, the only common environmental factor with a proven harmful effect.

The aim of this thesis was to describe the OC occurrence and familial aggregation among Danish twins and singletons. The purpose was to provide estimates for genetic counseling and provide a better understanding of the OC etiology.

Data from the Danish Facial Cleft Database, the Danish Twin Registry and the Danish Civil Registration System were used in the present thesis:

The *Danish Facial Cleft Database* comprises 10,025 OC cases born from 1936 to 2005. The treatment of OCs has, in Denmark, been centralized since the mid-1930s entailing high ascertainment of valid data. Information on cleft type, associated anomalies/syndromes, sex, and personal identification number was available. Each individual in the Danish Facial Cleft Database could be linked to the *Danish Twin Registry* (1870-2004) by use of the personal identification number which was kept in the *Danish Civil Registration System* (in existence since April 2, 1968) along with a link to more than 20,000 first degree relatives.

Initially, exact OC recurrence risk estimates for first cousins were provided for the first time. The recurrence risk was increased when compared to the background population, but to a lesser extent than the recurrence risk for second degree relatives of an OC individual, and this again was lower than for first degree relatives – approximately 0.2% : 0.5% : 0.7% : 3%, respectively. This pattern of recurrence along with recurrence risks stratified for severity, specificity, parent of origin effect, and family size for first degree relatives support the multifactorial threshold model of inheritance which, in 2008, was challenged by a Norwegian study. The results will help improve the counseling of family members of OC individuals.

With access to an isolated OC twin sample of 207 twins, three times the size of the previously exploited Danish sample (1970-1990), it was possible to further explore recurrence risk patterns, e.g. for twins discordant for oral clefting. The OC recurrence risk for offspring of twins discordant for oral clefting was compared and both risks were compared to the risk in the background population. The OC recurrence risk was the same whether the twin was affected or not, and it was increased compared to the background population. The similar recurrence for offspring of the affected and the unaffected twins indicated that both twins were carrying a genetic vulnerability for oral clefting. The results can be used in the rare genetic counseling situation of twin pairs

discordant for oral clefting. Furthermore, these results support the major role of genes in OC etiology and point to the need for a better understanding of OC sub-phenotypes.

Finally, the large OC twin sample made it possible to make a more powerful test of whether twinning has an effect on the risk of isolated OC by comparing the OC occurrence among twins and singletons. Also a more precise relative contribution of genes and the environment to the OC etiology was provided by use of probandwise concordance rates, tetrachoric correlations, and heritability estimates. No excess risk of OC for either all twins or monozygotic twins compared to singletons could be found. The highest degree of concordance was seen for the monozygotic twins (concordance rates were approximately 50% for monozygotic twins and 8% for dizygotic twins (p=0.01)) along with very high heritability estimates for both cleft lip with or without cleft palate and cleft palate (>90%). Moreover, for the first time it was indicated that dizygotic twins have an excess risk of OC compared to singleton siblings, which provides additional justification for the continuing search also for environmental factors of importance for the OC etiology.

8. Summary in Danish (Dansk resumé)

Læbe-ganespalte er blandt de hyppigste medfødte misdannelser og har betydelige sundhedsmæssige, sociale og økonomiske konsekvenser. Isoleret (non-syndromisk) læbe-ganespalte er en kompleks misdannelse, idet forudsætningerne for klassisk Mendelsk arvegang ikke er opfyldt, selvom misdannelsen ophobes i familier og har en betydelig genetisk komponent. Cirka 25% af de isolerede læbe-ganespalter kan tilskrives kendte genvarianter og cirka 5% kan tilskrives rygning på populationsniveau.

Formålet med denne afhandling var at beskrive forekomsten og familiær ophobning af læbe-ganespalte blandt danske tvillinger og enkeltfødte for at opnå estimater til brug i genetisk rådgivning samt give en bedre forståelse af årsagerne til læbe-ganespalte.

Data fra den Danske Læbe Ganespalte Database, Det Danske Tvillingeregister og Det Centrale Person Register danner grundlaget for denne ph.d. afhandling:

Den *Danske Læbe Ganespalte Database* omfatter alle personer født med læbe-ganespalte fra 1936 til 2005. Behandlingen af læbe-ganespalte har været centraliseret siden midten af 1930'erne i Danmark. Det har medvirket til en næsten komplet registrering i databasen af alle personer med læbe-ganespalte i Danmark. På disse personer er der oplysninger om spaltetype, tilstedeværelse af andre associerede anomalier/syndromer, om køn og personnummer. Hver enkelt person i den Danske Læbe Ganespalte Database kunne kobles til det *Danske Tvillingeregister* (1870-2004) ved hjælp af individernes personnummer. Personnumrene er blevet tildelt alle danskere via det *Centrale Person Register* siden 2. april 1968 sammen med en kobling til alle førstegradsslægtninge.

I det første studie var det muligt for første gang at give et præcist mål for gentagelsesrisikoen for læbe-ganespalte for fætre og kusiner. Gentagelsesrisikoen var øget i forhold til baggrundsbefolkningen, men i mindre omfang end gentagelsesrisikoen for andengradsslægtninge til individer med læbe-ganespalte, som igen var mindre end for førstegradsslægtninge – henholdsvis ca. 0,2%: 0,5%: 0,7% : 3%. Dette gentagelsesrisikomønster samt gentagelsesrisikoen for førstegradsslægtninge stratificeret for sværhedsgrad, specificitet, betydning af hvilken forælder der havde misdannelsen og familiens størrelse, støtter den multifaktorielle tærskelmodel, som på baggrund af en norsk undersøgelse i 2008 var blevet draget i tvivl. Disse resultater vil kunne bidrage til at forbedre rådgivningen af familiemedlemmer til personer med læbe-ganespalte.

Antallet af tvillinger med læbe-ganespalte er blevet øget med en faktor tre i forhold til tidligere studier af danske tvillinger med læbeganespalte (1970-1990). Det har gjort det muligt at undersøge flere gentagelsesrisikomønstre, som for eksempel tvillinger, der er diskordante for læbe-ganespalte. Gentagelsesrisikoen for børn af de diskordante tvillinger blev sammenlignet og begge risici blev sammenlignet med risikoen i baggrundsbefolkningen. Gentagelsesrisikoen var den samme uanset om tvillingen havde misdannelsen eller ej, og risikoen var forøget i forhold til baggrundsbefolkningen, hvilket kunne tyde på at begge tvillinger bærer en genetisk sårbarhed for

læbe-ganespalte. Disse resultater vil kunne bruges til genetisk rådgivning af tvillinger, der er diskordante for læbe-ganespalte, om end det er en sjælden situation. Desuden støtter disse resultater at generne spiller en stor rolle med hensyn til årsagerne til læbe-ganespalte og peger på behovet for en bedre forståelse af subtyper af læbe-ganespalte.

Afslutningsvis har det store antal tvillinger med læbe-ganespalte gjort det muligt med stor styrke at teste om det at være tvilling har betydning for risikoen for at fødes med læbe-ganespalte. Dette blev undersøgt ved at sammenligne læbe-ganespalteforekomsten blandt tvillinger og enkeltfødte. Desuden var det muligt at give et mere præcist mål for gener og miljøs relative betydning for læbe-ganespalte ætiologien ved hjælp af probandvise konkordansrater, tetrachoriske korrelationer, og heritabilitet (arvelighed). Der blev ikke fundet nogen overhyppighed af læbe-ganespalte for hverken alle tvillinger eller for enæggede tvillinger sammenlignet med forekomsten blandt enkeltfødte. Den højeste grad af konkordans blev set hos enæggede tvillinger (konkordansraten var ca. 50% for enæggede tvillinger og 8% for toæggede tvillinger (p = 0,01)). Heritabiliteten var høj for både læbespalte med eller uden ganespalte og ganespalte alene (>90%). Endvidere var det for første gang muligt at påvise at toæggede tvillinger har en overhyppighed af læbe-ganespalte i forhold til almindelige søskende, hvilket understøtter nødvendigheden af en fortsat søgen efter miljømæssige faktorer af betydning for læbe-ganespalte.

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Papers

Paper I

Grosen D, Chevrier C, Skytthe A, Bille C, Mølsted K, Sivertsen A, Murray JC, Christensen K. **A cohort study of recurrence patterns among more than 54,000 relatives of oral cleft cases in Denmark: support for the multifactorial threshold model of inheritance.**

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Dorthe Grosen, Cécile Chevrier, Axel Skytthe, et al.

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A cohort study of recurrence patterns among more than 54 000 relatives of oral cleft cases in Denmark: support for the multifactorial threshold model of inheritance

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ABSTRACT

Objectives To determine if the anatomical severity of oral clefting affects familial recurrence in a large population based sample. To provide reliable recurrence risk estimates for oral cleft for first, second, and third degree relatives.

Design Population based cohort study.

Setting Denmark.

Participants 6776 individuals affected with an oral cleft born from 1952 to 2005 and 54 229 relatives.

Main outcome measures Recurrence risk estimates for oral cleft for first, second, and third degree relatives and stratification by severity, specificity, parent of origin effect, and family size for first degree relatives.

Results For cleft lip and palate probands we observed recurrence risks for first, second, and third degree relatives of respectively 3.5% (95% CI 3.1% to 4.0%), 0.8% (95% CI 0.6% to 1.0%), and 0.6% (95% CI 0.4% to 0.8%). Individuals affected by the most severe oral cleft had a significantly higher recurrence risk among both offspring and siblings, eg, the recurrence risk for siblings of a proband with isolated bilateral cleft lip with cleft palate was 4.6% (95% CI 3.2 to 6.1) versus 2.5% (95% CI 1.8 to 3.2) for a proband born with a unilateral defect.

Conclusions Anatomical severity does have an effect on recurrence in first degree relatives and the type of cleft is predictive of the recurrence type. Highly reliable estimates of recurrence have been provided for first cousins in addition to more accurate estimates for first and second degree relatives. These results and the majority of prior data continue to support a multifactorial threshold model of inheritance.

INTRODUCTION

Oral clefting is one of the most frequent congenital malformations, with a birth prevalence of one to two per 1000 live births varying by ancestral origin.[1] Despite corrective surgery, being born with an oral cleft has lifelong implications for those affected and their families.[2] Therefore, there is a continuing need for a better understanding of the aetiology and the mechanism of clefting in order to improve the counselling of families at increased risk and to identify aetiologic factors that may suggest improvements in therapy or prevention.

The aetiology of oral clefting is complex, with both genes[1 3–12] and the environment playing important roles.[13–19]

Oral clefts are commonly subdivided into two phenotypically and aetiologically distinct groups: cleft lip with or without cleft palate, and the cleft palate only.[20 21] Cleft lip with or without cleft palate can be further subdivided into cleft lip only and cleft lip with cleft palate. Cleft lip and cleft lip with cleft palate may be aetiologically distinct or represent a continuum of severity, with cleft lip with cleft palate being the more severe form of the defect.[22] Cleft lip with or without cleft palate can be incomplete or complete depending on the involvement of the alveolus (primary palate) and the length of the cleft in the palate (submucous cleft palate or cleft in the soft palate only versus cleft in both the soft and the hard palate). Either sub-phenotype can be associated with major physical or developmental anomalies and/or be a part of a recognised syndrome. In these cases the oral cleft is classified as a syndromic cleft as opposed to an isolated or non-syndromic cleft. Isolated clefts can, however, be associated with minor associated anomalies. A wide range of the frequency of syndromic clefts has been reported in the literature: 10–30% for cleft lip with or without cleft palate, and 20–60% for cleft palate only.[23 24]

Since the early 1950s clinical practice has been to counsel parents of a child born with a cleft on the risk of having a subsequent child with an oral cleft, using empiric recurrence risks consistent with the multifactorial threshold model of inheritance.[25–27] This model has been challenged by several complex segregation analysis studies, but there has never been sufficient evidence to reject the model.[28 29] Since the 1990s several studies of both recurrence patterns as well as the identification of specific loci or genes contributing to clefts have ruled out a single, major locus model and the multiplicative additive or independent loci models. This leaves us with the best fitting model of inheritance being multiple genes interacting in a multiplicative manner which agrees with the multifactorial threshold model.[5 6 30–36] A recent study using a single, well defined population from Norway has challenged the multifactorial threshold model since they found no effect of severity on inheritance.[37] If this result can be replicated in additional and larger studies it would have substantial implications for the clinical counselling of families and the understanding of the underlying causes of clefting.[38]

This Danish study on more than 54 000 relatives provided not only the opportunity to examine this

possible paradigm shift, but also the opportunity for the first time to estimate the recurrence risk for first cousins (third degree relatives) and notably improve the accuracy of the existing recurrence risk estimates on first and second degree relatives to an individual with an oral cleft.

METHODS

The present study is a population based cohort study based on record linkage between three nationwide, population based registers in Denmark.

The Civil Registration System was established in April 1968 and it registers all individuals alive and residing in Denmark since then. All individuals have a unique 10 digit personal identification number. This register also includes identifiers that link all first degree relatives (parents and siblings). These identifiers allow construction of sibships (by matching individuals with parental personal identification numbers) which can be linked using parent sibships to form complex pedigrees. On the maternal side links have been almost complete (96%) since 1959, but for individuals born before 1952 it is considerably lower (46%). A similar pattern is apparent for the paternal personal identification numbers although the availability tends to be slightly lower (92% post-1959 and 39% pre-1952).

The Danish Facial Cleft Database now encompasses the 1936 to 2005 cohort. It includes 10 022 live born individuals born with an oral cleft of which 9143 (91.4%) individuals are registered by a personal identification number. Two nationwide ascertainment sources are used to ascertain the Danish individuals with oral cleft. For the earlier birth cohorts (1936 to 1987) patient lists were used, maintained by Dr Poul Fogh-Andersen from 1934 to 1986 and since then continued at the Rigshospitalet where all surgical treatment has been centralised since 1986. At the two National Institutes for Defects of Speech, where treatment other than surgical may occur, all reports from the midwives on children born with an oral cleft have been kept since 1954. Oral clefts, mainly submucous cleft palate, recognised later in a child's life are also reported to the institutes. The ascertainment is very high for the complete cohort, and capture-recapture methods have indicated 99% ascertainment for the sub-phenotype isolated cleft lip with or without cleft palate in the period 1983 to 1987.[39] In the Danish Facial Cleft Database overt oral clefts are classified into three groups: cleft lip, cleft lip with cleft palate, and cleft palate. Both cleft of the lip only and cleft of the lip and the primary palate is considered a cleft lip phenotype. A distinction is not possible in this study, which is also the case with regards to completeness of the cleft lip when it occurs together with cleft palate. Cleft lip with or without cleft palate can be subdivided into unilateral and bilateral clefts, with unilateral clefts being the mildest form and the bilateral the most severe form of cleft. The cleft palate phenotype includes the range of submucous cleft palate being the mildest form, to cleft in the soft palate only (the intermediate form) to the most severe form, cleft in the hard and soft palate. Bifid uvula is considered a microform of cleft palate. Recently it has been suggested that orbicularis oris muscle defects and dental anomalies can also be considered microforms of oral cleft. Expanding the phenotypes of oral clefting will greatly improve future genetic studies, but in this study it has not been possible to take the microforms into account due to incomplete ascertainment.[40–44]

In the Danish Facial Cleft Database, 876 (9.6%) of the individuals born with an oral cleft are registered as also having at least one non-cleft major anomaly or a recognised syndrome. Malformations such as neural tube defects were designated as major anomalies. Defects such as polydactyly were considered minor malformations. Minimal defects such as nevi were not considered associated anomalies. The classification of the associated anomalies into minor and major has been maintained to maintain consistency in the Danish Facial Cleft database since it has been used from the inception of the registry.[1] It is based on whether the anomaly is likely to be part of a syndrome. For the earlier birth cohorts from 1936 to 1987 the number of individuals born with either an associated major anomaly or a syndrome was likely underestimated,[39] but for the later birth cohorts medical records were reviewed by Bille *et al* in 2005 to obtain more complete information about associated anomalies/syndromes.[45 46] The recorded number of associated anomalies/syndromes are slightly lower in the Danish population compared to other populations,[23 47] but the pattern with more anomalies/ syndromes associated with cleft palate compared to cleft lip with or without cleft palate is the same. Table 1 shows the frequency of the syndromic oral clefts according to the cleft phenotypes and the time period observed in the Danish Facial Cleft Database.

The Danish Twin Registry includes the birth cohorts from 1870 to 2004 corresponding to more than 80 000 twin pairs. The twins are all born in Denmark and they were ascertained independently of any disease. Before 1968 the ascertainment was about 90%, but since the establishment of the Civil Registration System it has been considered complete.[48] Zygosity determination on same sex twins has been validated and the misclassification rate has been found to be <5%.[49 50] About 75% of the twins in the registry have an assigned zygosity. In the Danish Twin Registry overall 85% of the twins are registered with a personal identification number, and since 1968 100% of the twins have a personal identification number that enables linkage to the Civil Registration System, hence linkage to relatives of an individual with an oral cleft can be established.

Study population

For the present study the population was restricted to all live born individuals with a valid personal identification number in the Civil Registration System. The children were born in Denmark between 1952 and 2005 and were registered with an isolated cleft lip, cleft lip with cleft palate, or cleft palate only with no recognised syndrome or non-cleft major malformation. Individuals born before 1952 were excluded since their records in the Civil Registration System were unlikely to include parental links. We made an exception for the grandparents of the probands so that grandparents born from 1936 to 2005 were included, but only if the intervening parent was born between 1952 and 2005.

Operationally, the probands from the Danish Facial Cleft Database were first linked to the Civil Registration System using their personal identification numbers. Because the Civil Registration System allowed the identification of the parents, full and half siblings, offspring, grandparents, full and half nieces/ nephews, full and half aunts/uncles, and cousins for each proband, we were able to count the total number of affected and unaffected relatives of each cleft type. Different sets of files were created with the proband or the parents of the proband as the index case. Finally the Danish Twin Registry was linked to the Danish Facial Cleft Database in order to identify twin pairs of whom at least one of the twins was affected with an oral cleft. Using the described procedure, several relatives were identified more than once through one proband. For example, a woman with two siblings, each of whom had a child with an oral cleft, could be included as an aunt twice. In our computations of the recurrence risk such individuals were only counted once.

The recurrence risk was estimated by dividing the number of affected relatives of type *R* (R=parents, offspring, etc) by the

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Table 1 Frequency of individuals affected by an oral cleft according to phenotypes, time period, and sex from the Danish Facial Cleft Database (1936—2005)

	Oral clefts			CL			CLP			CP		
	Total	With major birth defects/ syndromes (%)	Sex ratio (M:F)	Total	With major birth defects/ syndromes (%)	Sex ratio (M:F)	Total	With major birth defects/ syndromes (%)	Sex ratio (M:F)	Total	With major birth defects/ syndromes (%)	Sex ratio (M:F)
1936—1951	1,524	16 (1.1)	1.4	491	3 (0.6)	1.8	601	4 (0.7)	2.4	432	9 (2.1)	0.6
1952—1961	1,268	44 (3.5)	1.5	401	2 (0.5)	1.7	497	20 (4.0)	2.2	370	22 (5.9)	0.8
1962—1971	1,541	104 (6.7)	1.6	435	9 (2.1)	1.8	599	33 (5.5)	2.4	507	62 (12.2)	1.0
1972—1981	1,455	135 (9.3)	1.5	416	5 (1.2)	2.0	516	41 (7.9)	2.1	523	89 (17.0)	1.0
1982—1991	1,289	172 (13.3)	1.5	350	15 (4.3)	1.7	456	42 (9.2)	2.3	483	115 (23.8)	0.9
1992—2001	1,548	314 (20.3)	1.3	411	17 (4.1)	1.6	557	93 (16.7)	2.0	580	204 (35.2)	0.8
2002—2005	518	91 (17.6)	1.6	159	8 (5.0)	2.0	201	25 (12.4)	2.1	158	58 (36.7)	0.9
1952—2005	**7,619**	**860 (11.3)**	**1.5**	**2172**	**56 (2.6)**	**1.8**	**2826**	**254 (9.0)**	**2.2**	**2621**	**550 (21.0)**	**0.9**
1936—2005	**9,143**	**876 (9.6)**	**1.5**	**2663**	**59 (2.2)**	**1.8**	**3427**	**258 (7.5)**	**2.2**	**3053**	**559 (18.3)**	**0.8**

CL, cleft lip; CLP, cleft lip with cleft palate; CP, cleft palate.

total number of relatives R. The risk to full siblings is therefore equivalent to the 'singles' method described by Davie (1979)[51] under complete ascertainment. We also estimated the recurrence risk among later born siblings and for full siblings according to family size. Probands and siblings who were members of a twin pair were not included in these estimates. For all other types of relatives, twins were included as both probands and relatives in order to keep the groups as comparable as possible. We computed the relative risk (λ) for a type of relative R of affected individuals compared with the background population by dividing the recurrence risk to a relative R by the population prevalence.[5 6]

For relatives of the three groups of probands—isolated cleft lip, isolated cleft lip with cleft palate, and isolated cleft palate—we have provided the estimates of the recurrence risk of all types of oral clefts. For first degree relatives we also provided the recurrence risks for different degrees of severity, for the same or dissimilar types of isolated oral clefts, according to family size, and with respect to parent of origin effect. We graded the bilateral clefts as more severe than unilateral for both cleft lip with cleft palate and for cleft lip only. For the cleft palate cases, submucous cleft palate was graded as the mildest form and involvement of both the hard and soft palate as the most severe form.

Heterogeneities between risks were computed from the Pearson's χ^2 test or from the exact test using mid p approach when numbers were very small.[52]

A total of 3 703 337 live births were registered in Denmark during the period 1952 to 2005. The analyses were carried out on 2116 isolated cleft lip probands, 2572 isolated cleft lip with cleft palate probands, and 2088 isolated cleft palate only probands.

The Intercooled Stata 9.2 and SAS software (version 9.1) were used for all computations.

RESULTS

Unless specifically noted, all of the results and discussion are for isolated oral clefts.

The population prevalence of oral clefts in Denmark for the period 1952 to 2005, including associated anomalies/syndromes, was 2.1 per 1000 live births.

Among the 9143 individuals affected by an oral cleft registered in the Danish Facial Cleft Database from 1936 to 2005 we observed two cleft lip with or without cleft palate cases for each cleft palate only case (table 1). Approximately 2% of cleft lip, 8% of cleft lip with cleft palate, and 18% of the cleft palate only cases were associated with one or more major anomalies or syndromes. In the youngest birth cohorts these proportions had increased to approximately 5%, 12%, and 37%, respectively. We observed a predominance of males in the cleft lip and cleft lip with cleft

palate groups and an excess of females in the cleft palate group, all in accordance with previous studies.

Recurrence risk for first, second, and third degree relatives

The results of the recurrence risk (absolute and relative risk (λ)) for relatives of individuals affected by a cleft lip, cleft lip with cleft palate, or cleft palate only are shown in table 2. The recurrence risk for siblings of the cleft lip with cleft palate probands was estimated to 3.9% (95% confidence interval (CI) 3.2% to 4.7%) and it was comparable to the estimate for the later-born siblings of 4.6% (95% CI 3.5% to 5.8%). The risk of cleft lip with cleft palate for the offspring was 4.1% (95% CI 3.2% to 5.1%) and also similar to the risk for the siblings. The risk to parents, however, was 2.5% (95% CI 1.8% to 3.1%); this was significantly lower than the risk to either of the two other groups of firs -degree relatives. The relative risk of cleft lip with cleft palate for all first degree relatives was 17 (95% CI 15 to 19) times higher than the risk observed in the background population.

Recurrence risk was estimated for four types of second degree relatives: half siblings, nieces/nephews, aunts/uncles, and grandparents; they were lower than the risk to first degree relatives and yet quite similar to each other. The risk of cleft lip with cleft palate for second degree relatives was four (three to five) times higher than the risk observed in the background population.

Recurrence risks were estimated for three types of third degree relatives: first cousins, half nieces/nephews, and half aunts/uncles. The risks were all lower than the risks to second degree relatives and were quite similar to each other. The risks of cleft lip with cleft palate for third degree relatives were three (two to four) times higher than the risk observed in the background population.

The same pattern was found for the other two cleft types for all three kinds of relatives.

For first cousins in particular the recurrence risk estimates for the three cleft types were indistinguishable (table 2). The overall estimate of the recurrence risk for oral cleft for first cousins was 0.4% (95% CI 0.3% to 0.6%), ie, two (1.5 to 2.7) times higher than in the background population.

Recurrence risk by severity, specificity, parent of origin effect, and family size for first degree relatives

The recurrence risk stratified by severity for siblings—eg, from bilateral cleft lip with cleft palate to bilateral cleft lip with cleft palate—of 4.6% (95% CI 3.2% to 6.1%) shows that the most severe cleft type for both cleft lip and cleft lip with cleft palate tends to recur. The only exception from that pattern was for cleft

Table 2 Risks of oral cleft for first, second, and third degree relatives according to the probands' three phenotypes of cleft (Denmark, 1952—2005)

	First degree relatives					Second degree relatives						Third degree relatives			
	Siblings†					Half-siblings†							Half	Half	
	Offspring	All	Subsequent	Parents	All	All	Subsequent	Nieces/ nephews	Aunts/ uncles	Grand- parents‡	All	First cousins	nieces/ nephews	aunts/ uncles	All
iCL probands (n=2116)															
Total number	1439	2442	1162	1861	5742	810	362	2155	2213	1729	6907	3532	544	468	4544
No. affected*	50	60	25	47	157	8	3	20	13	4	45	11	3	1	15
Risk (%)	3.5	2.5	2.2	2.5	2.7	1.0	0.8	0.9	0.6	0.2	0.7	0.3	0.6	0.2	0.3
Relative risk (λ)	17	12	10	12	13	5	4	5	3	1	3	2	3	1	1.6
(95% CI)	(13 to 22)	(9 to 15)	(7 to 15)	(9 to 16)	(11 to 15)	(2 to 9)	(0.8 to 10)	(3 to 7)	(2 to 5)	(0 to 2)	(2 to 4)	(0.8 to 3)	(0.5 to 7)	(0.0 to 4)	(0.9 to 3)
iCLP probands (n=2572)															
Total number	1591	2954	1389	2209	6754	938	454	2702	2742	2093	8475	4303	649	547	5499
No. affected*	65	116	64	55	236	5	3	22	29	10	66	22	6	5	33
Risk (%)	4.1	3.9	4.6	2.5	3.5	0.5	0.7	0.8	1.1	0.5	0.8	0.5	0.9	0.9	0.6
Relative risk (λ)	20	19	22	12	17	3	3	4	5	2	4	2	4	4	3
(95% CI)	(15 to 25)	(16 to 23)	(17 to 28)	(9 to 16)	(15 to 19)	(0.8 to 5)	(0.6 to 8)	(2 to 6)	(3 to 7)	(1.1 to 4)	(3 to 5)	(1.6 to 4)	(2 to 9)	(1.4 to 9)	(2 to 4)
iCP probands (n=2088)															
Total number	1211	2379	1171	1820	5410	828	396	2002	2175	1550	6555	3344	478	521	4343
No. affected*	51	78	39	38	167	8	5	22	12	7	49	15	2	4	21
Risk (%)	4.2	3.3	3.3	2.1	3.1	1.0	1.3	1.1	0.6	0.5	0.7	0.4	0.4	0.8	0.5
Relative risk (λ)	20	16	16	10	15	5	6	5	3	2	4	2	2	4	2
(95% CI)	(15 to 26)	(13 to 20)	(12 to 22)	(7 to 14)	(13 to 17)	(2 to 9)	(2 to 13)	(3 to 8)	(1 to 4)	(0.9 to 4)	(3 to 5)	(1.2 to 3)	(0.2 to 6)	(1.0 to 8)	(1.5 to 3)

Prevalence of oral clefts in the background population born in Denmark between 1952 and 2005: (7619)/(3 703 337)=0.21%.

CIs are computed from $C*(\sqrt{a} \pm \frac{1}{2} Z_{\alpha/2})^2/n$, where a=number of affected relatives of type R, n=total number of relatives of type R, α=0.05 and C=100 for the CI of the risk in percentage and C=(1/prevalence in the background population) for the CI of the relative risk.

*Number of relatives affected by an oral cleft (including syndromic oral cleft and oral cleft with associated anomalies).

†For computation of the recurrence risks for siblings, twins are excluded from both groups of the probands and their siblings; the numbers of iCL, iCLP and iCP probands are respectively 2055, 2487 and 2044.

‡Grandparents of probands are born between 1936 and 2005.

iCL, solated cleft lip; iCLP, isolated cleft lip with cleft palate; iCP, isolated cleft palate.

palate only where the moderate severity (from soft cleft palate to soft cleft palate) had the highest recurrence risk (3.9%, 95% CI 2.5% to 5.6%), although not statistically different from the severest form (table 3). We observed the same pattern for subsequent siblings (results not shown). Using data from the Norwegian study,[37] reclassified in order to be comparable to our severity classification, we found a consistent pattern of repeating the most severe cleft type for all cleft types, including cleft palate (table 4). No statistically significant heterogeneity between the recurrence risks was seen within each phenotype. The same pattern was seen for offspring (results not shown).

The recurrence risk within each subtype of oral cleft for siblings—eg, from cleft lip with cleft palate to cleft lip with cleft palate—showed a consistent pattern of recurrence specificity with the highest recurrence risk to the same subtype within all three subtypes (table 5). The same pattern was found for subsequent siblings and offspring but with less statistical power (data not shown). For the two known distinctly different defects—cleft lip with or without cleft palate, and cleft palate—we found a crossover risk that was significantly lower than the recurrence risk within the type but slightly higher than the risk in the background population (eg, for cleft palate to cleft lip with cleft palate 0.2%, 95% CI 0.0% to 0.4%).

We estimated the recurrence risk for the offspring stratified by whether the relatives were on the maternal or paternal side of the case. For the cleft lip and cleft lip with cleft palate that is predominant in males, we found the highest recurrence risk for children when the mother was affected, and for the cleft palate with female predominance we found the highest recurrence risk for children when the father was affected (table 6). Within each phenotype the recurrence risks were, however, not statistically significant.

The recurrence risk according to family size for full siblings (1787 siblings, 44 affected) showed the same pattern of increasing risk with an increasing number of children in a family for all sub-phenotypes—eg, for cleft lip with cleft palate the recurrence risk increased from 2.0% (95% CI 1.2% to 2.9%) in a family with two children to 6.5% (95% CI 1.2% to 16.0%) in a family with four children. Though not statistically significant, the direction of the point estimate is clear.

DISCUSSION

For the siblings and offspring we found that severity does have an effect on the recurrence risk for oral clefting, with the only statistically non-significant exception for moderate cleft palate

Table 3 Recurrence risk for siblings of having the same phenotype of cleft as the probands according to laterality or severity of clefting (Denmark, 1952—2005)

			Recurrence for siblings		
Phenotype	Sub-phenotype	Total number of siblings	Number	Risk (%) (95% CI)	p (heterogeneity)*
iCL probands	Unilateral	1977	27	1.4 (0.9 to 1.9)	0.50
	Bilateral	205	4	2.0 (0.5 to 4.3)	
iCLP probands	Unilateral	1963	49	2.5 (1.8 to 3.2)	0.004
	Bilateral	854	39	4.6 (3.2 to 6.1)	
iCP probands	Sub-mucous CP	659	18	2.7 (1.6 to 4.1)	0.75
	Soft CP	622	24	3.9 (2.5 to 5.6)	
	Soft—hard CP	999	26	2.6 (1.7 to 3.7)	

11%, 5% and 5% of siblings for respectively the iCL, the iCLP and the iCP probands are not included in these numbers because of unknown sub-phenotype of the probands.

Twins are excluded from both groups of the probands and their siblings.

*Pearson χ^2 test.

iCL, solated cleft lip; iCLP, isolated cleft lip with cleft palate; iCP, isolated cleft palate.

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Table 4 Recurrence risk for subsequent siblings of having the same phenotype of cleft as the probands according to laterality or severity of clefting (Norway from Sivertsen et al[37])

Phenotype	Sub-phenotype	Total number of siblings	Recurrence for siblings		p (Heterogeneity)*
			Number	Risk (%) (95% CI)	
iCL probands	Unilateral	189	1	0.5 (0.0 to 2.1)	0.23
	Bilateral	24	1	4.2 (0.0 to 16.3)	
iCLP probands	Unilateral	173	4	2.3 (0.6 to 5.1)	0.18
	Bilateral	65	4	6.2 (1.6 to 13.7)	
iCP probands	Submucous CP	22	0	—	0.17
	Soft CP	84	2	2.4 (0.2 to 6.8)	
	Soft-hard CP	71	4	5.6 (1.5 to 12.5)	

Compared to the published data,[37] we excluded stillbirths and minor anomalies from both groups of the probands and their subsequent siblings.
*Exact test using mid p approach, specific for very small numbers.
iCL, isolated cleft lip; iCLP, isolated cleft lip with cleft palate; iCP, isolated cleft palate.

severity. We found complete specificity of the recurrence risk within the distinct cleft types. As have others, we found that cleft lip and cleft lip with cleft palate occur more frequently in males than females whereas there is a female excess with cleft palate.[53] Affected mothers have the highest risk of passing on cleft lip and cleft lip with cleft palate and the affected fathers have the highest risk of passing on cleft palate.

Our recurrence risk estimates on first, second, and third degree relatives are in good agreement with the recurrence risk estimates on our first report on a smaller subset of the Danish population for the isolated oral clefts born between 1952 and 1987.[5 6] The precision of the estimates has been increased notably and has benefited from an increase in sample size by a factor of 10 for numbers of phenotyped relatives. For the first time it is possible to present reliable estimates for first cousins (and other third degree relatives) of individuals affected by a cleft lip, cleft lip with cleft palate, or cleft palate.

Due to the use of record linkage from the highly reliable Danish national registers instead of self recorded family history, and the fact that the Danish population is well defined and genetically homogeneous, our study avoids common limitations such as the grouping of all oral clefts together or incomplete ascertainment.[38]

The hallmarks for multifactorial inheritance are: (1) most affected children have normal parents; (2) recurrence risk increases with the number of affected children in a family; (3) recurrence risk increases with severity of the defect;

Table 5 Specificity of the recurrence risks for siblings (Denmark, 1952—2005)

Phenotype	Total number of siblings	Recurrence for siblings					
		Number			Risk (%) (95% CI)		
		iCL	iCLP	iCP	iCL	iCLP	iCP
iCL probands	2442	35	24	0	1.4 (1.0 to 1.9)	1.0 (0.6 to 1.4)	—
iCLP probands	2954	22	87	4	0.7 (0.5 to 1.1)	2.9 (2.4 to 3.6)	0.1 (0.0 to 0.3)
iCP probands	2379	0	4	67	—	0.2 (0.0 to 0.4)	2.8 (2.2 to 3.5)

Twins are excluded from both groups of the probands and their siblings.
iCL, isolated cleft lip; iCLP, isolated cleft lip with cleft palate; iCP, isolated cleft palate.

Table 6 Recurrence risk for offspring of having the same phenotype of cleft as the probands by the gender of the affected relative (Denmark, 1952—2005)

Phenotype	Gender	Total number of offspring	Recurrence for offspring		p (Heterogeneity)*
			Number	Risk (%) (95% CI)	
iCL probands	Male	865	14	1.6 (0.9 to 2.6)	0.27
	Female	574	14	2.4 (1.3 to 3.9)	
iCLP probands	Male	993	19	1.9 (1.1 to 2.9)	0.11
	Female	598	19	3.2 (1.9 to 4.8)	
iCP probands	Male	456	15	3.3 (1.8 to 5.2)	0.35
	Female	755	18	2.4 (1.4 to 3.6)	

*Pearson χ^2 test.
iCL, isolated cleft lip; iCLP, isolated cleft lip with cleft palate; iCP, isolated cleft palate.

(4) consanguinity slightly increases the risk for an affected child; (5) risk of affected relatives falls off very quickly with the degree of relationship; and (6) when the two sexes have a different probability of being affected, the least likely sex, if affected, is the most likely sex to produce an affected offspring.[54]

In the present study we observed a higher recurrence risk among offspring and siblings compared to parents, a tendency to repeat the same cleft type in the recurrence, a strong effect on recurrence according to severity, a steep drop-off in the recurrence risk from first to second degree relatives and from second to third degree relatives, and the highest recurrence risk in the least frequently affected sex. We also observed a tendency towards an increasing recurrence risk for full siblings with an increased number of sibs, but the results did not reach formal significance. All these results support the multifactorial threshold model of inheritance; hence our data do not support a shift away from the use of the multifactorial threshold model of inheritance. This model has been in use since the 1960s[25—27] and, despite several challenges,[28 29 37] it still appears to be the best model to explain the aetiology of oral clefting.[5 6 30—36]

The recent study from Norway challenged this model. The Norwegian analysis included stillbirths and syndromic forms of clefting with the isolated forms and pooled cleft lip only cases with cleft lip with cleft palate cases. It found no effect of severity on the recurrence risk using a detailed classification system different from the one used in Denmark for cleft lip. For cleft palate the classifications were the same.[37] When the Norwegian data were reanalysed using a similar strategy to the one in this report (ie, with exclusion of stillbirths and associated malformations and syndromes, the distinction between cleft lip with cleft palate and cleft lip only, and the use of the same classification of severity as the one reported here), we found that the observed values of recurrence risks according to cleft severity are consistent with the expectations under the multifactorial threshold model (table 4). We therefore find that the Norwegian results and the results presented here using a larger (three times the size) population based sample on a cohort from a neighbouring country do not contradict each other.

A few factors may, however, contribute to a slight underestimation of our recurrence risk estimates. First is the lack of personal identification numbers on the 6% that we excluded in order to be able to do the linkage to the Civil Registration System for all probands. To exclude any selection bias on this behalf we did the analysis for the 1968—2005 birth cohorts (results not shown) in which fewer personal identification numbers are missing (3%), and the point estimates remained virtually unchanged. Another factor that may have biased our results towards an underestimation is the fact that only legally identifiable parental links are used in the Civil Registration

System, so in the case of adoption or non-paternity the children cannot be identified. In the Danish Facial Cleft Database, however, only individuals born in Denmark are included. According to the national Statistics Denmark, adoptive children comprise a maximum of 1.5% of all the birth cohorts in the present study, and about 90% of them are born outside of Denmark, hence are excluded in the Danish Facial Cleft Database.[55] Any bias from this is likely to be minimal.

In general the ascertainment is very high for oral clefts in Denmark, but the ascertainment of cleft palate is slightly lower due to the milder forms being asymptomatic until development of speech or even longer, but when diagnosed they are reported to the speech institutes. Due to the 70 year long follow-up period in the Danish Facial Cleft Database, selection bias due to this late entry of the cleft palates is likely to be minimal.[39 56]

To some extent there is differential misclassification in the earlier birth cohorts in the Danish Facial Cleft Database, since individuals with undiagnosed associated anomalies or syndromes can be misclassified as individuals with isolated oral clefts. Yet this only concerns the milder forms of associated anomalies/syndromes since the most severe cases were ascertained in connection with surgery. The slight increase in the crossover risk between clefts involving the lip and those involving the palate only could be explained by the chance occurrence of syndromic clefts or by genes like *MSX1* and *IRF6* where both types of clefts may appear in the same family and with no additional phenotypic traits to result in it being assigned to a syndrome category. Since the initiation of the update of the Danish Facial Cleft Database from 1988 to 2005 the ascertainment and classification of associated anomalies/syndromes have been enhanced considerably.[45] The analyses based on truncated periods, such as the 1968 to 2005 birth cohorts, provided similar results to those of the present study, so we believe that this bias is likely to be minimal.

We did not expect a higher prevalence of oral cleft among those who married persons from the oral cleft cohort. We did indeed observe very few affected spouses, 1.5 per 1000, which is a little less than the population frequency; hence it is unlikely to influence the present results of familial recurrence risk patterns. In addition, the Danish population is in general known to be both homogeneous and to have a low incidence of consanguinity among ethnic Danes.[57]

The recurrence risk for siblings might be biased if parents had fewer children than expected after having a first child born with oral cleft. If that is the case, the risk for all siblings would be underestimated and different from the risk for the later born siblings. We computed both risks and the results support no such assumption.

In the present study, for each sub-phenotype of oral cleft and for each grouping of relatives of individuals affected by an isolated oral cleft, we chose to present the recurrence risks to

What is already known on this topic?

► The aetiology of oral cleft is complex with respect to both genes and environment.
► The recurrence risk is increased for both first and second degree relatives but results for third degree relatives have been inconclusive.
► A recent study showed no impact of anatomical severity on the recurrence risk of oral cleft.

What this study adds?

► Anatomical severity does have an effect on recurrence in first degree relatives and the type of cleft is predictive of the recurrence type.
► The recurrence risk is increased for third degree relatives by a factor of 2 compared to the background population.
► The results support a multifactorial threshold model of inheritance and provide important knowledge to affected family members and the persons who counsel them.

oral cleft of any kind. These estimates were expectedly higher than the estimates of the recurrence risks to isolated oral cleft.

Although cleft lip alone and cleft lip with cleft palate have been considered the same—both embryological and epidemiological—since the work of Fogh-Andersen[20] in the 1940s, increasing evidence, including the work reported here, suggests that important differences may be present. Earlier, Harville et al[22] presented evidence of epidemiological differences in cleft lip only cases and molecular data for differences have also been recently published.[19] In the molecular case a common variant in a *TFAP2A* binding site in the enhancer regions of the *IRF6* gene has one allele that strongly predisposes families to isolated cleft lip only (odds ratio ∼3) but has little effect on cleft lip with cleft palate. The effect acts in populations of different geographic origin and has an attributable fraction of 18% in Danish and Norwegian cases. This coupling of epidemiological and molecular findings, as well as new data on the role of sub-phenotypes such as orbicularis oris defects in clefts[43] or evolving data from genome wide associations studies of clefting,[8] will enable more specific studies of aetiology as well as the ability to provide more family specific recurrence risks in the future.

In conclusion, these analyses benefit from the very high quality of the Danish population based data sources in which biases are likely to be minimal and the large sample size has allowed us to provide very reliable estimates. Our results are consistent with the majority of studies done on oral cleft recurrence which support the multifactorial threshold model as the best explanation of the inheritance of oral clefting, and are consistent with a recent study when the same variables are analysed.

We have substantially improved the precision of the estimate of the recurrence risk for the Danish population and for the first time we have provided estimates for first cousins. This study will improve the counselling of individuals with an oral cleft or relatives of an individual with an oral cleft. Some similarities between different populations can be shown, as in the current study between the Danish and the Norwegian population, but the Danish population also shows some significant genetic and environmental differences from other populations. Thus these results should be replicated in other populations to improve their generalisability. It also supports the search for aetiologic factors based on specific cleft type and that different factors (genes or variants within the same genes) may be relatively more active in cleft lip alone versus cleft lip with cleft palate versus cleft palate alone.

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Papers

Paper II

Grosen D, Bille C, Pedersen JK, Skytthe A, Murray JC, Christensen K. **Recurrence Risk for Offspring of Twins Discordant for Oral Cleft – A Population-based Cohort Study of the Danish 1936-2004 Cleft Twin Cohort.**

Recurrence Risk for Offspring of Twins Discordant for Oral Cleft

- A Population-based Cohort Study of the Danish 1936-2004 Cleft Twin Cohort

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ABSTRACT

Objective: To estimate the recurrence risk of isolated oral cleft (OC) for offspring of the unaffected co-twins of OC discordant twin pairs and to compare this risk to the recurrence risk in the offspring of the affected co-twin as well as to the risk in the background population.

Design: A Danish population-based cohort study

Participants: During 1936-2004, 207 twin pairs were ascertained, among whom at least one twin had an OC. The index persons were twins discordant for OC who had children (N=117), and their offspring (N=239). The participants were ascertained by linkage between The Danish Facial Cleft Database, The Danish Twin Registry and The Danish Civil Registration System.

Main outcome measures: OC recurrence risk for offspring of the affected and unaffected twin and relative risk compared to the background prevalence.

Results: Among 110 children of the 54 OC affected twins, two (1.8%) children had OC corresponding to a significantly increased relative risk (RR = 10; 95% CI 1.2 to 35) when compared to the frequency in the background population. Among the 129 children of the 63 unaffected twins, three (2.3%) children were affected, corresponding to a significantly increased relative risk (RR = 13; 95% CI 2.6 to 36) when compared the background prevalence.

Conclusion: In OC discordant twin pairs similar increased recurrence risks were found among offspring of both OC affected and OC unaffected twins. This provides further evidence for a genetic component in cleft etiology and is useful information for genetic counseling of twin pairs discordant for clefting.

Key Words: recurrence risk; oral cleft; cleft lip and palate; twins; genetics

INTRODUCTION

Nonsyndromic oral clefts (OCs) are among the most common congenital malformations and have substantial implications for the affected individuals and their families. The etiology is multifactorial with both genetic and environmental factors playing an important role [Vieira, 2008]. Most individuals born with OC have unaffected parents, even though family data suggest a very high heritability (greater than 70%) [Christensen et al., 1993a]. This genetic influence entails an increased relative risk ranging from 15 times higher for first-degree relatives to two times higher for third-degree relatives compared to the risk of the background population [Grosen et al., 2010].

Twins with OC provide unique research possibilities. While it has been speculated that twinning might disturb the normal process of development of the lip and palate in the fetus during early pregnancy, there is no compelling evidence for an effect of twinning on the risk of OC [Christensen et al., 1993a; Christensen et al., 1993b; Christensen et al., 1996a; Mitchell et al., 1997]. In addition, twinning may affect (or occur secondary to) epigenetic phenomena such as X inactivation or DNA methylation that could also play a role in developmental disruptions such as clefting [Kimani et al., 2007]. Finally, discordant twins (one twin affected) could arise from somatic genetic events such that the affected twin might be the only member of the pair carrying a specific risk allele [Kondo et al., 2002; Mansilla et al., 2005; Sakuntabhai et al., 1999].

The question of how best to counsel the unaffected twin in a twin pair discordant for OC was raised by Wyszynski et al. in case reports from 1996 and 2002 [Wyszynski et al., 1996; Wyszynski et al., 2002]. At that time no empirical data were available, but the authors speculated that the risk for the offspring of the unaffected co-twin in the pair would be three to ten times higher than the risk of the background population, i.e.

potentially as high a risk as for the affected twin, but likely to be smaller since the co-twin was unaffected.

The aim of the present study was to use the large population-based registers available in Denmark to estimate the empiric recurrence risk for offspring of unaffected twins in twin pairs discordant for nonsyndromic OC and to compare this risk to the risk of the affected twins and the background population (Figure 1).

MATERIAL AND METHODS

The present study was a population-based cohort study based on record linkage between three nationwide registers in Denmark.

The Civil Registration System could identify all individuals who resided in Denmark at any time since its establishment in April, 1968. All individuals had a unique ten-digit personal identification number. The personal identification number included date of birth and information on sex, and contained a built-in check code disclosing invalid numbers. The register also contained a link to all first-degree relatives enabling a construction of pedigrees showing legal familial relationships (by matching individuals who share parental personal identification numbers).

The Danish Facial Cleft Database encompassed the birth cohorts from 1936 to 2005 and contained 10,025 live born individuals with OC in Denmark. For 9,146 (91%) individuals, a valid personal identification number was available. The nine percent without a personal identification number was OC individuals who either died before 1968 or could not be uniquely identified in order to be assigned a personal identification number. Both the registration and the treatment of individuals with OC have been centralized in Denmark since the 1930s and this has entailed a very high ascertainment for the cohorts under study. Clefts discovered later in a child's life were also registered [Bille et al., 2005a]. Capture-

recapture methods have indicated a 99% ascertainment for the sub-phenotype isolated cleft lip with or without cleft palate (CL/P) in the period 1983 to 1987 [Christensen et al., 1992]. Overt oral clefts could be classified into three groups in the Danish Facial Cleft Database: cleft lip (CL), cleft lip with cleft palate (CLP) and cleft palate only (CP). For bifid uvula the ascertainment was low and microforms of oral clefts, such as defects in the orbicularis oris muscle or dental anomalies, were not routinely registered in the Danish Facial Cleft Database. In the Danish Facial Cleft Database 876 (9.6%), individuals were registered with OC and another major anomaly and/or a recognized syndrome. The pattern of more anomalies associated with CP and fewer with CL and CLP was consistent with other cleft populations, but the overall rate was lower in Denmark. The OC association with anomalies/syndromes has previously been described in greater detail [Bille et al., 2005c; Bille et al., 2005a; Bille et al., 2005b; Christensen, 1999; Grosen et al., 2010].

The Danish Twin Registry included the Danish birth cohorts from 1870 to 2004, corresponding to more than 80,000 twin pairs. The twins were all born in Denmark, and they were ascertained independently of any disease. Before 1968 the ascertainment was about 90%, but since the establishment of the Civil Registration System it has been considered complete [Skytthe et al., 2002]. Zygosity determination has been made on same sex twins by use of questionnaires to determine the degree of similarity between co-twins and has been validated by comparison of blood type determinants and genetic markers. The misclassification rate was less than 5% [Bonnelykke et al., 1989; Christiansen et al., 2003], and about 75% of the twins had an assigned zygosity. In the Danish Twin Registry, 85% of the twins were registered with a personal identification number, and since 1968, 100% of the live born twins have been assigned a personal identification number which enables a linkage to the Civil Registration System, and hence linkage to the offspring of the twins.

Study population

During 1936-2004, 207 mono- and dizygotic twin pairs were born in Denmark, among whom at least one twin was affected by an isolated OC (CL, CLP or CP). The index cases were twins discordant for isolated OC who had children (N=117), and their offspring (N=239) born from 1956 to 2005 (Figure 2). The population was restricted to all live born individuals with a valid personal identification number in the Civil Registration System to make it possible to identify these individuals in both the Danish Facial Cleft Database and the Danish Twin Registry. Only isolated OCs were included, that is, individuals with OC but with no other major anomalies or recognized syndromes; hence, unless specifically noted, all the results and discussion are for isolated OCs.

Technically, the Danish Facial Cleft Database and the Danish Twin Registry were linked by the personal identification number identifying twin pairs of whom at least one twin was affected with an isolated OC. Using the Civil Registration System offspring of the twins were identified and again linked to the Danish Facial Cleft Database to identify any offspring also affected by an isolated OC.

The recurrence risk for children of the affected twins was estimated by dividing the number of affected offspring by the total number of offspring. The risk was similar to the risk found when using the "singles" method described by Davie [1979] under complete ascertainment. For offspring of the unaffected twin in the discordant twin pairs, the same method was used when computing a pseudo-recurrence risk, even though an OC could technically not recur for an unaffected twin. The relative risks were compared to the prevalence in the background population born in the same time period, by dividing the recurrence risk for offspring by the population prevalence [Christensen et al., 1996b; Mitchell et al., 1996]. Fisher's exact test was used to compare recurrence risk between

affected and unaffected twins and the background population frequency. Stratification for type of OC was not possible for the recurrence risk estimates due to small sample size.

To describe our twin population (N=414 twins), the comparison between the likelihood of the unaffected and affected twins of becoming a parent and of the number of children born was performed using a Poisson regression. For the number of children born, the period of observation in adulthood for each twin was taken into account. We compared the age at first birth using a linear regression. Twin concordance, sex of the twins, zygosity, and OC were included as confounders when relevant. STATA 10.1 was used for all computations and the "cluster" option was used in all regression models to correct for the correlated nature of the twin data.

RESULTS

Table I shows the number of OC affected twins and unaffected co-twins according to phenotype and zygosity.

Of the 207 twin pairs included in the study, 185 twin pairs were discordant for OC and 22 pairs were concordant (Figure 2). Among the discordant twins, 117 (32%) twin individuals had reproduced compared to 6 (14%) twins among the concordant twins (p = 0.05).

Recurrence and relative risk for discordant twins

The population frequency of OC in Denmark from 1956 to 2005 was 1.8 per 1,000 live births. Among the 110 children of the 54 OC twins, two (1.8%) children had OC, corresponding to a significantly increased relative risk (RR = 10; 95% confidence interval (CI) 1.2 to 35) when compared to the frequency in the background population (Table II). Among the 129 children of the 63 unaffected twins, three (2.3%) children were affected, corresponding to a significantly increased relative risk (RR = 13; 95% CI 2.6 to 36) when compared to the background prevalence. Both estimates were in the same order of magnitude

as the relative risk (RR = 19; 95% CI 17 to 22) for the recurrence risk in the general population compared to the background prevalence.

The proportion of monozygotic parents to all offspring of the discordant twins and to the recurrent cases was computed from the numbers displayed in Figure 2. Of all the 117 discordant twins with children, 19 (16%) were monozygotic, but two of the five (40%) twins with children who also had an OC were monozygotic (p = 0.20).

Table III displays the OC recurrence risk and the relative risk for unaffected and affected twins, respectively, stratified for zygosity. The monozygotic affected twin parents had no affected offspring. The highest recurrence risk was seen for offspring of the monozygotic unaffected twins and the relative risk was significantly increased (RR = 42; 95% CI 5.3 to 140) when compared to the background population.

When the OC recurrence risk for the unaffected monozygotic twins was compared to the OC recurrence risk for the unaffected but dizygotic twins, the relative risk was increased (RR = 7.9; 95% CI 0.75 to 85), although not statistically significant. For the dizygotic twins, the relative risk for the affected twins was increased (RR = 2.3; 95% CI 0.21 to 25) when compared to the unaffected dizygotic twins, but also statistically non-significant.

All of the 414 twins (affected and unaffected) were followed for roughly the same number of reproductive years (i.e. from age 15 to 45 for both male and female, no fathers were older than 45). The unaffected twins had a significantly increased likelihood of having children (odds ratio = 1.3; 95% CI 1.1 to 1.6) when compared to the OC affected twins. When adjusting for possible confounding variables (concordance and sex), the estimate showed the same direction but was no longer statistically significant. Having an OC had no effect on the number of children per parent (incidence rate ratio = 1.2 (95% CI 0.92 to 1.5)). The parental age of twins who reproduced (N=123) followed a Gaussian distribution and the distribution of the time under study was the same whether the twins were affected by

OC or not. No difference in age at first birth was seen according to OC status or zygosity, but female twins were 20 (95% CI -0.21 to 40) months younger at birth of the first child compared to the male twins.

DISCUSSION

This national population-based cohort study found that the OC recurrence risk for offspring of twins discordant for OC was similar regardless of whether the twin was affected, i.e. the unaffected twin's risk for an affected offspring was not significantly different from that of the affected twin. In addition, the recurrence risk for offspring of both the affected and unaffected twin from a twin pair discordant for OC was significantly increased compared to the background population frequency.

We have previously estimated recurrence risks for OC for more than 50,000 first-, second- and third-degree relatives by use of the same database, but for the 1952 to 2005 cohort [Grosen et al., 2010]. This study adds information on twin recurrence to be used in genetic counseling (Figure 3).

Monozygotic twins share 100% of their genes as opposed to 50% for dizygotic twins. Since the etiology of OC is mainly genetic, for a monozygotic twin pair discordant for OC, we would expect both twins to be carrying susceptibility genes for OC. The affected twin could have an additional novel mutation acquired after the division of the zygote. That mutation may have pushed this twin over the threshold of developing OC. When reproducing, both twins would pass on susceptibility genes, but since the one twin exceeded the threshold, we expected this twin's risk to be the highest [Wyszynski et al., 1996; Wyszynski et al., 2002]. We observed the highest risk for offspring of the unaffected twins relative to the background population risk, although the confidence intervals were wide. This indicates that offspring of the unaffected co-twin of a discordant pair have an increased liability to OC and likely a similar liability as offspring of affected twins. Heritability studies

suggest that OC has a strong genetic component [Christensen et al., 1993a; Murray, 2002]. The unaffected twins may therefore also have been carrying susceptibility genes for OC like the affected twins were most likely to have done. Another explanation could be that both twins had been exposed to an environmental factor while in the womb, which later increased the risk for their offspring when they reproduced.

In addition, we found similar OC recurrence risks for twin offspring and offspring to affected parents from the background population. It could indicate that the mechanism of clefting is the same whether the parent was a twin or not. A direct comparison of the OC occurrence among twins and singletons would be more suited to answer that question. Previous results have been ambiguous, but the majority found similar OC prevalence for twins and singletons [Christensen et al., 1993a; Christensen et al., 1993b; Christensen et al., 1996a; Mitchell et al., 1997; Nordstrom et al., 1996; Robert et al., 1996].

Our study of recurrence risk among twins is the largest to date owing to the long standing ascertainments in the Danish Facial Cleft Database and the Danish Twin Registry. The study covers a complete country with 70 years of follow up for the twins and 50 years for their offspring. Nonetheless, the study still lacks sufficient power to make valid conclusions when stratifying for zygosity and types of OC. We found that the overall recurrence risk was higher among offspring of the unaffected twins despite inclusion of the dizygotic twins. When we analyzed our results stratified by zygosity, we found that the highest risk was for offspring of the unaffected monozygotic twins and that risk was eight times higher than the risk for offspring of the unaffected dizygotic twins. Along with a tendency towards an increased proportion of monozygotic twin parent to recurrent cases compared to the proportion of monozygotic twin parents to all offspring, it was most likely the recurrence risk for offspring of monozygotic twins that drove the overall recurrence risk estimate for unaffected twins. These results add further evidence for a genetic etiology of

oral clefting, but do not rule out yet unmeasured environmental factors. The power issue was critical as the event of clefting and twinning co-occurring in the time period observed was one in 45,000 live births in Denmark. Hence, when stratification was made for zygosity, caution should be taken when interpreting these results. Similar data from other Nordic countries could provide an additional source for obtaining estimates stratified for both zygosity and cleft phenotype.

Since the early work of Fogh-Andersen, the phenotypes CL/P and CP have been considered embryologically and epidemiologically distinct defects [Fogh-Andersen, 1942], and the two phenotypes rarely run in the same families. Accordingly, in the present study, the recurrence was of a similar type (CL/P or CP) except in one family which could represent an undiagnosed case (born in 1946) of van der Woude syndrome where both CL/P and CP occur. If this one family was excluded, the same overall results were obtained: the recurrence risk for offspring of unaffected twins was 1.6% (95% CI 0.19 % to 5.6 %) which did not differ from the recurrence risk among affected twins of 1.8% (p = 1.0), and a significantly increased relative risk (RR = 8.7; 95% CI 1.1 to 31) when compared to the background prevalence.

In the Danish Facial Cleft Database, ascertainment for cohorts born before 1954 depended mainly on surgical files [Christensen et al., 1992]. An individual with OC had to survive until the age of 2 months to be evaluated for surgery and hence be included in the later established Danish Facial Cleft Database. This survival bias was especially an issue for the twins and individuals with a severe OC and/or an associated anomaly/syndrome, who had an even higher neonatal mortality particularly before the availability of neonatal intensive care in the 1960s. Individuals with milder forms of CP that could easily be overlooked or might not need surgery were prone to selection bias [Bille et al., 2005a; Christensen, 1999]. Since 1954, when midwives in Denmark were required to report all

types of OC discovered at birth or later in life directly to the National Institute for Defect of Speech, survival bias was reduced to a minimum, and ascertainment has been close to complete[Christensen et al., 1992]. This possible selection bias for the earlier cohorts could have resulted in a slight underestimation of the recurrence risk estimate, but comparison between the twins and the background prevalence should not be affected.

Previous studies have indicated a small effect of paternal age [Bille et al., 2005b] on OC occurrence, and our unpublished results have shown a slightly different reproductive history pattern for individuals affected by OC compared to the reproductive history pattern of the background population. For our twin population, the likelihood of having children was slightly decreased for individuals affected with OC. OC had no influence on parity for those having children or on the age of birth of the first child. All results were as expected from population figures from the complete Danish Facial Cleft population (unpublished results).

In 2002 Kondo et al reported on a pair of monozygotic twins discordant for van der Woude syndrome in which oral clefting is a major manifestation. They sequenced a large section on chromosome 1 that had been identified through genetic linkage studies and found a mutation in the *IRF6* gene that is now known to be the cause of van der Woude syndrome. This strategy had been used for another Mendelian disorder [Sakuntabhai et al., 1999] and was subsequently applied to OC. These studies have used monozygotic twins discordant for isolated OC, but have failed to identify differences in genes of importance for oral clefting [Kimani et al., 2009; Mansilla et al., 2005], differences in copy number variations [Kimani et al., 2009; Mansilla et al., 2005] or in X-chromosome inactivation patterns [Kimani et al., 2007]. The major difference between the two disorders is that van der Woude syndrome is a monogenic disease whereas isolated OC is a multifactorial trait. Our results provide an additional explanation as to why this otherwise reasonable twin approach continues to fail.

The unaffected twins were carrying a liability for oral clefting, e.g., susceptibility genes for oral clefting. They had, however, not reached the threshold for developing an overt cleft, either by chance or due to low penetrance or variable gene expression as seen for *IRF6*. Mutations in *IRF6* can result in tooth agenesis for some individuals and isolated clefts or syndromic forms of clefts for other individuals [Vieira, 2008]. Likewise, the twins could have had a microform of OC such as a defect in the orbicularis oris muscle and hence not routinely registered. Several studies have shown that microforms of OC are seen more frequently among unaffected relatives, and a large multicenter study is currently exploring this finding in greater detail [Chatzistavrou et al., 2004; Marazita, 2007; Neiswanger et al., 2007; Weinberg et al., 2006; Weinberg et al., 2008a; Weinberg et al., 2008b]. Future studies including the microforms of OC can both increase the study population and diminish the risk of missing a true association between genes and OC occurrence.

In conclusion, this study benefits from the use of reliable Danish registers where selection bias was reduced to a minimum. It included an entire country for 70 years, but still sample size was a limiting factor. The similar increased recurrence risks found among offspring of both cleft-affected and cleft-unaffected discordant twins contribute further support for a genetic component in cleft etiology. Finally, this information is useful in the rare genetic counseling situation of twin pairs discordant for clefting.

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Fig 1. Pedigree of a family with monozygotic/dizygotic twin girls discordant for oral clefting.
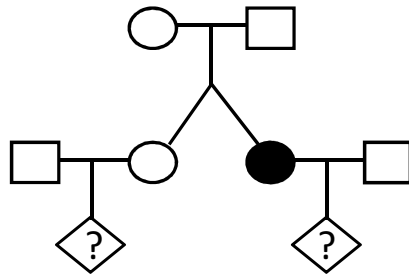
Fig 2. Twin recurrence of isolated oral cleft, twins born from 1936 to 2004. MZ=monozygotic, DZ=dizygotic, UZ=unknown zygosity, CL=Cleft Lip, CLP=Cleft Lip and Palate, CP=Cleft Palate.

Fig. 3. Pedigree with recurrence risk and 95% confidence intervals. A: Family with twin girls discordant for oral cleft, B: affected parents (twins and singletons), C: background population.

A: 2.3% (0.48 – 6.7) and 1.8% (0.22 – 6.4)   **B**: 3.5% (3.1 – 4.0)   **C**: 0.18%

TABLE I. Number of twins from twin pairs (N=207) with at least one twin affected by an isolated oral cleft

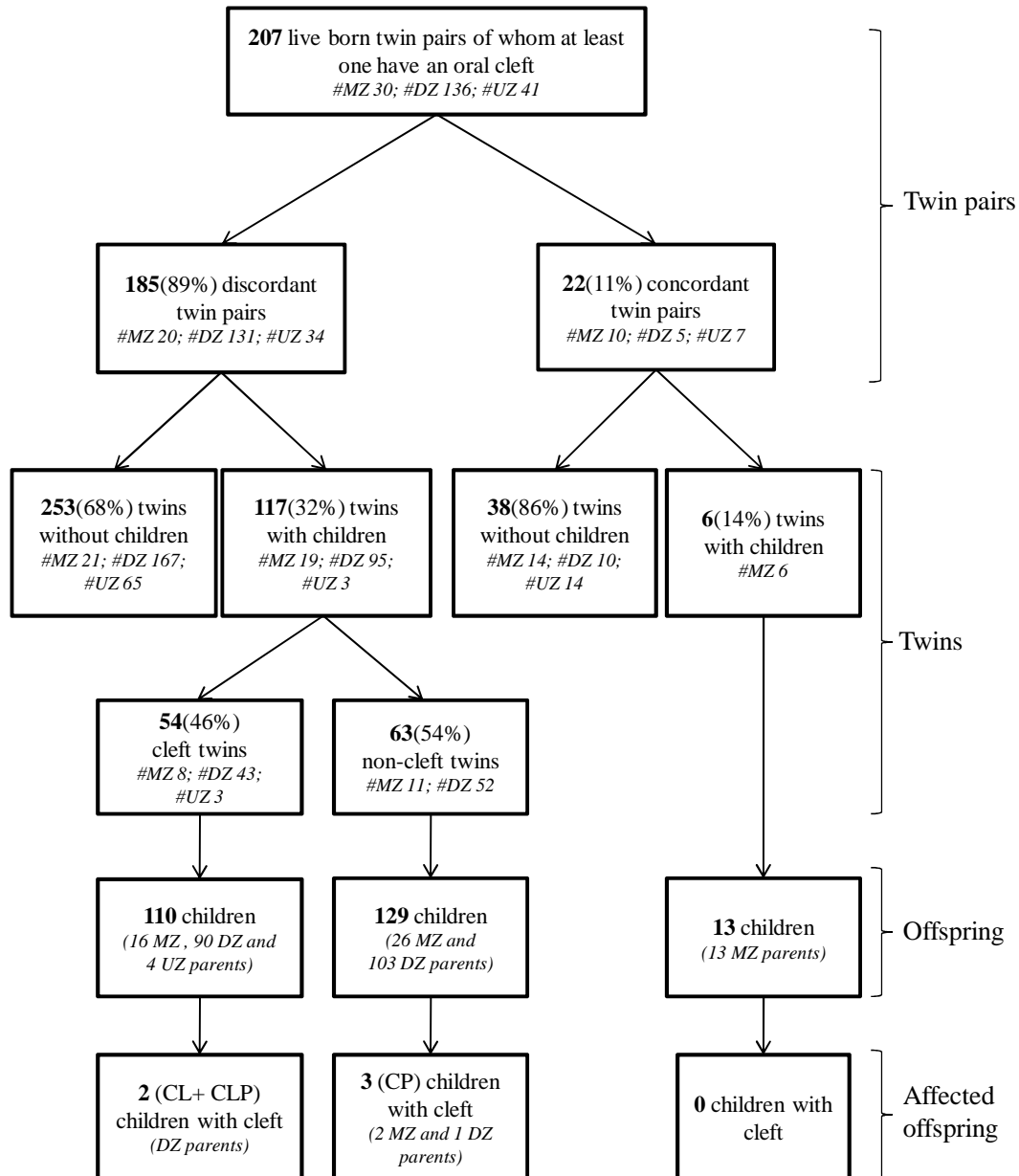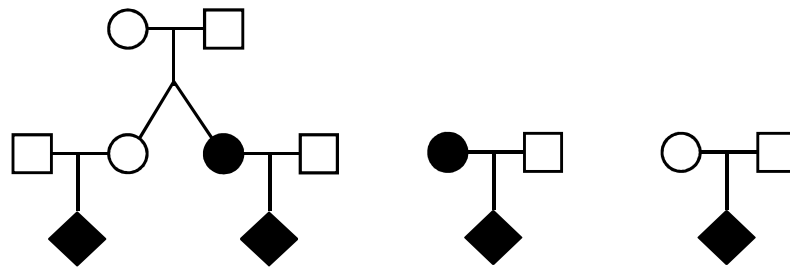| Phenotype | Cleft twin population (1936-2004) | | | |
	Monozygotic (%)	Dizygotic (%)	Unknown zygosity (%)	All zygosities
Cleft lip	20 (27)	39 (53)	15 (20)	74
Cleft lip with cleft palate	12 (12)	68 (68)	20 (20)	100
Cleft palate	8 (14)	34 (62)	13 (24)	55
No oral cleft	20 (11)	131 (71)	34 (18)	185
All	60 (14)	272 (66)	82 (20)	414

TABLE II. Recurrence and relative risk of isolated oral cleft, Denmark 1956 - 2005

Designation of Relationship	Number Affected (n)	Total (N)	Recurrence Risk (%) [95% confidence interval]	Relative Risk*, [95% confidence interval]
Background population prevalence	6,194	3,394,923	0.18	Reference
Offspring of affected parents (background population)	234	6,642	3.5 [3.1 ; 4.0]	**19 [17 ; 22]**
Offspring of affected discordant twins	2	110	1.8 [0.22 ; 6.4]	**10 [1.2 ; 35]**
Offspring of non-affected discordant twins	3	129	2.3 [0.48 ; 6.7]	**13 [2.6 ; 36]**

Significant if p < 0.05, in bold
*Compared to the risk in the background population born in the same time period

TABLE III. Twin pairs discordant for isolated oral cleft. Zygosity stratification of recurrence risk and relative risk, Denmark 1956 to 2005

| Twin parents status | | Recurrence | | Recurrence risk (%) [95% confidence interval] | Relative risk*, [95% confidence interval] |
Zygosity	Oral Cleft	Number Affected (n)	Total (N)		
Monozygotic	No	2	26	7.7 [0.95 ; 25]	**42 [5.3 ; 140]**
	Yes	0	16	0	0
Dizygotic	No	1	103	0.97 [0.025 ; 5.3]	5.3 [0.17 ; 29]
	Yes	2	90	2.2 [0.27 ; 7.8]	**12 [1.7 ; 43]**

Significant if p<0.05, in bold
*Compared to the risk in the background population born in the same time period

Papers

Paper III

Grosen D, Bille C, Petersen I, Hjelmborg JvB, Pedersen JK, Skytthe A, Murray JC, Christensen K. **Twins with Oral Cleft in Denmark 1936-2004.**

Type of manuscript: Original Article

Title: Twins with Oral Cleft in Denmark 1936 – 2004

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Running head:

Authors: Grosen et al.

Title: Twins with Oral Cleft in Denmark

Abstract

Background: Studies of the effect of twinning on oral cleft etiology is ambiguous.

Methods: This national population-based cohort study investigated whether twinning was associated with isolated oral cleft and estimated the twin probandwise concordance rate and heritability. Twins (207 affected/130,710) and singletons (7,766 affected/4,798,526) born from 1936-2004 in Denmark were ascertained by linkage between the Danish Facial Cleft Database, the Danish Twin Registry and the Civil Registration System. Oral cleft prevalence and prevalence proportion ratio (PPR) for twins versus singletons was computed and stratified for three sub-phenotypes. Probandwise concordance rates and heritability for twins were estimated for cleft lip with or without cleft palate and cleft palate.

Results: The prevalence of oral cleft was 15.8 per 10,000 for twins and 16.6 per 10,000 for singletons (PPR = 0.95; 95% confidence interval 0.83 to 1.1). For monozygotic and dizygotic twins similar oral cleft PPR's were found. A higher probandwise concordance rate was found for cleft lip with or without cleft palate for monozygotic twins compared to dizygotic twins (50 % vs. 7.7%, respectively). For cleft palate a similar pattern was found. The recurrence risk for both cleft lip with or without cleft palate and cleft palate in dizygotic twins was found to be different from the recurrence risk for non-twin siblings. For both phenotypes the corresponding heritability estimates were above 90%.

Conclusion: No excess risk of oral cleft could be demonstrated for twins compared to singletons. The concordance rates and heritability estimates show a strong genetic component in oral cleft etiology.

Key Words: twins, prevalence, concordance rate, heritability, cleft lip and palate

INTRODUCTION

Oral clefts, including cleft lip, cleft lip with cleft palate and cleft palate only are among the most common congenital malformations. The three sub-phenotypes most likely have overlapping but also distinct etiologies.[1] Non-syndromic oral clefts are complex traits since they exhibit no classical Mendelian inheritance, but show strong familial aggregation and have a substantial genetic component.[2-5] About 25% of the isolated oral clefts can be explained by known genes and approximately 5% by smoking, the only common environmental factor with a proven harmful effect.[6,7]

Several studies have compared the oral cleft occurrence in twins and singletons. The results are ambiguous and most studies are limited by small sample size, ascertainment bias, inclusion of syndromic forms of oral cleft, and a lack of zygosity information.[8-18] So far, the majority of data have not provided compelling evidence of oral cleft to be associated with twinning in general or with monozygotic twinning in particular.[4,5,19-22]

The relative contribution of genetic and environmental factors to the oral cleft etiology has previously been estimated by use of classical twin studies on a small Danish twin population born from 1970-1990.[4,5] The probandwise concordance rate for monozygotic twins were about 60% and between 0 to 10% for dizygotic twins with corresponding heritability estimates of approximately 70%. However, the estimates were hampered by the small sample size[23] especially for cleft palate. It has not been possible either in the Danish population or in any other population to determine whether dizygotic twins have an excess risk of oral cleft compared with ordinary siblings as might be expected if environmental factors influence this risk.

The aim of the present study was to determine whether twinning was associated with isolated oral cleft by comparing the oral cleft occurrence among twins and singletons in a sample of

twins three times the size of samples in previous studies, and to provide estimates of the oral cleft probandwise concordance rate for monozygotic and dizygotic twins as well as heritability estimates.

METHODS

Study population

In this population-based twin study, individuals with an oral cleft identified through the Danish Facial Cleft Database[23,24] from the 1936-2004 birth cohorts could be linked to the Danish Twin Registry.[25] The linkage between the population-based registries was enabled by means of the unique personal identification number assigned by the Civil Registration System. When the Civil Registration System was established in 1968, everyone alive or residing in Denmark at that point in time in addition to all live births since then was assigned such a personal identification number, allowing most individuals to be tracked several decades back in time.

The Danish Facial Cleft Database encompasses the birth cohorts from 1936 to 2005 and contains 9,146 individuals with a valid personal identification number. Both the registration and the treatment of individuals with oral cleft have been centralized in Denmark since the 1930s and this has entailed an almost complete ascertainment for the cohorts under study. Clefts discovered later in a child's life were also registered.[24,26] Capture-recapture methods have indicated a 99% ascertainment for the sub-phenotype isolated cleft lip with or without cleft palate in the period 1983 to 1987.[26] For phenotypes other than cleft lip, cleft lip with cleft palate, and cleft palate, the ascertainment was low; hence the microforms (bifid uvula, defects in the orbicularis oris muscle etc.) of oral cleft were excluded from the study. Excluded also were the syndromic forms of oral cleft and oral cleft cases with other major anomalies. Minor malformations including polydactyly or hip dislocation were included. In the present paper all cases refer to isolated oral cleft unless

otherwise specified. The Danish Facial Cleft Database has previously been described in greater detail for both ascertainment and anomalies.[23,24]

The Danish Twin Registry comprises more than 80,000 twin pairs born in Denmark since 1870. The twins were ascertained independently of any disease. The overall ascertainment of live born twins from 1930 and onwards was about 80%. Since the establishment of the Civil Registration System, the ascertainment has been considered complete for live born twins, and since 1973 for all twins.[25] Zygosity determination of same sex pairs has been made through four standard questions about physical resemblance, a method with less than 5% misclassification for the birth cohorts 1900-1982.[27] Zygosity determination on twins with oral cleft was made using the same method. Here also the misclassification was estimated to be less than 5%.[28] About 75% of the twins in the register have an assigned zygosity. Information on zygosity is only accessible through the Danish Twin Registry.

To assess the number of twins obtained through the Danish Twin Registry summary data were extracted from the *Statistics Denmark*[29] where many aspects of life for all residents of Denmark have been collected for administrative purposes on a consistent basis. Since the establishment of the Civil Registration System, it has been possible to track individuals by use of their personal identification number, but before 1968 the data were aggregated.

Of the 130,710 twins and 4,798,526 singletons born from 1936 to 2004, 207 twins and 7,966 singletons were born with an isolated oral cleft.

Statistics

Oral cleft prevalence and prevalence proportion ratio (PPR) for twins versus singletons stratified for sex and the sub-phenotypes cleft lip, cleft lip with cleft palate, and cleft palate were estimated for the 1936 to 2004 cohorts by use of data from Statistics Denmark.[29] For the 1968 to 2004 cohorts, further stratification was made for zygosity by use of data from the Danish

Twin Registry. Differences in the monozygotic:dizygotic proportion in the oral cleft twin population was compared to the total twin population. The distribution of monozygotic, dizygotic same sex, dizygotic opposite sex, and unknown zygosity was compared between the cleft lip, cleft lip with cleft palate, and cleft palate individuals. All binary comparisons were made using Fisher's exact test or, whenever possible, Poisson regression, in order to take into account the correlated nature of the twins by use of the cluster function in Stata 10.1 (StataCorp, USA) and to test for interaction between sex and zygosity.

The relative contribution of genes and environment to the oral cleft etiology was estimated by use of both the probandwise concordance rates, the tetrachoric correlation (corresponding to the intraclass correlation for a continuous outcome), and heritability for cleft lip with or without cleft palate and cleft palate. Twin pairs born from 1936 to 2004 were included. The basic assumption is that the environment of monozygotic and dizygotic twins is similar, and therefore, any differences in their concordance rates must be attributable to their differences in genetic similarity (monozygotic twins share 100% of their genes and dizygotic, on average, 50 % of the parental genetic pool). The probandwise concordance rate is an estimator of the probability that one twin has an oral cleft given that the co-twin is affected. The oral cleft recurrence risk for full siblings (non-twins) is the number of affected siblings divided by the total number of siblings. Since the Probandwise concordance rate provides estimates of risk for the individual rather than for the pair, it can be directly compared to the recurrence risk for ordinary siblings, who are genetically equivalent to dizygotic co-twins.[30] This comparison offers the possibility to single out the effect of the environment since the number of shared genes is similar for both dizygotic twins and full siblings, but the twins shared the uterus whereas the siblings were present in the uterus at a different point in time. A change in the environment could either be caused by an intentional change in the mother's risk behavior in the subsequent pregnancy after having a first child with oral cleft, or a

change in other environmental factors that the mother was not in control over. The probandwise

concordance rate for monozygotic and dizygotic twins and the recurrence risk for siblings were

compared using exact statistical methods. All probandwise concordance rate comparisons were

verified by the use of bootstrapping taking into account that the prevalence of oral cleft for

monozygotic and dizygotic twins was the same.

Tetrachoric correlations for monozygotic and dizygotic twins (same and opposite sex) were

compared under the assumption of the multifactorial threshold model (liability threshold model)

which best describes the etiology of oral cleft.[31] A higher correlation for monozygotic twins

compared to dizygotic twins indicates that genetic factors contribute to the phenotypic variation.

The magnitude of the genetic contribution can be computed using heritability estimates which are

independent of the prevalence of the malformation studied. For the tetrachoric correlations and the

heritability estimates both same sex and opposite sex twin pairs were included, but thresholds were

not adjusted for effects of sex. The total variance *(V)* could be decomposed in $V = A + D + C + E$

where *A* refers to the additive genetic effects, *D* refers to the dominant genetic effect (intraloci

interaction), *C* refers to shared environmental effects and *E* refers to the unique environmental

effect. Univariate genetic models[32] were fitted to contingency tables using maximum likelihood

estimation with Mx statistical modelling.[33] First, a saturated model was fitted and thereafter the

following models were fitted: ACE, ADE, AE, CE and E. The best fitting model was chosen in

accordance with the lowest Akaike Information Criterion (AIC) (χ^2-2·df). Thereby both the

goodness of fit and the simplicity of the model were taken into account. The 95% confidence

intervals (CI) were calculated for the standardized parameter estimates (heritability) of the best

fitting model.

The Intercooled Stata 10.1 version (StataCorp, College station, TX, USA) was used for all computations except for the tetrachoric correlations and heritability estimates, where Mx (freeware from www.vcu.edu/mx/) was used.

RESULTS

Prevalence

Along with the prevalence, the number of twins and singletons according to sex and oral cleft phenotype from the Danish 1936 to 2004 cohorts are shown in Table 1. The prevalence of oral cleft was similar for twins and singletons (15.8 and 16.6 per 10,000, respectively, prevalence proportion ratio (PPR) = 0.95; 95% confidence interval 0.83 to 1.10). Twins (both male and female) were less likely to have cleft palate compared to singletons (PPR = 0.63; CI 0.53 to 0.76). Twin boys, however, seemed more likely to have cleft lip with cleft palate than singleton boys (PPR = 1.20; CI 1.01 to 1.42). The sex distribution was similar for twins and singletons. When stratifying into two time periods with a cut-point in 1968 corresponding to the establishment of the Civil Registration System, the oral cleft prevalence was lowest (PPR = 0.73; 95% CI 0.73 to 0.93) for the twins before 1968 due to much fewer cleft palates among the twins for both sexes. From 1968 to 2004, the oral cleft prevalence for twins and singletons were comparable (PPR = 1.15; 95% CI 0.95 to 1.38), but the twins had a higher prevalence of cleft lip with cleft palate compared to singletons (PPR = 1.43; 95% CI 1.09 to 1.90), driven by an excess of cleft lip with cleft palate among the twin boys (Table 2). There was no interaction between sex and zygosity. The Danish Twin Registry identified 110,556 of the 130,710 twins (85%) registered in Statistics Denmark in the complete time period, but the ascertainment was nearly complete (99%) from 1968 to 2004 (Table 1). For both twins and singletons, male preponderance for cleft lip and cleft lip with cleft palate and female

preponderance for cleft palate were found. The differences were, however, more pronounced for the twins.

The PPR for the oral cleft twin prevalence relative to the singleton prevalence is displayed in Table 2 stratified for sex, phenotype, and zygosity for the 1968 to 2004 cohorts based on data from the Danish Twin Registry. The key prevalence ratios based on the numbers from Tables 1 and 2 are summarized in Figure 1. Comparable PPRs were found for monozygotic twins relative to singletons for all of the oral cleft phenotypes. The same pattern was seen for the dizygotic twins with the exception of the twins with cleft lip with left palate in whom the prevalence was increased (PPR = 1.57; 95% CI 1.13 to 2.20) due to an increased prevalence for the dizygotic twin boys (PPR = 1.78; 95% CI 1.23 to 2.59). A similar pattern was seen for twins with unknown zygosity (oral cleft PPR = 1.08; 95% CI 0.79 to 1.49).

Similar monozygotic:dizygotic twin proportions were found for the cleft twin population and the total twin population, 1:3.9 and 1:3.5, respectively. Likewise, the proportion of monozygotic, dizygotic same sex, dizygotic opposite sex, unknown zygosity twins were similar for cleft lip, cleft lip with cleft palate or cleft palate individuals.

Probandwise concordance rates, tetrachoric correlations, and heritability

The probandwise concordance rate for monozygotic twins, all dizygotic twins, the subset of same sex dizygotic twins and twins with unknown zygosity was stratified into cleft lip with or without cleft palate and cleft palate, and is displayed in Table 3 along with the recurrence risk for ordinary siblings. For cleft lip with or without cleft palate, evidence was provided for the probandwise concordance rate for monozygotic twins of 50% being higher than the probandwise concordance rate of 7.9% for the dizygotic twins. For cleft palate the same pattern with a higher probandwise concordance rate for monozygotic twins (33%) compared to dizygotic twins (7.4%)

were found. The oral cleft recurrence risk estimates for siblings were derived from the Danish 1952-2005 cohorts[31]. When comparing the probandwise concordance rate for dizygotic twins to the recurrence risk for ordinary siblings, the probandwise concordance rate was highest for both phenotypes. When stratified for sex and the three sub-phenotypes, the confidence intervals were wide, but the pattern was very consistent with a probandwise concordance rate range for monozygotic twins from 33% to 67%, for dizygotic twins from 6% to 12% and for unknown zygosity twins from 13% to 33%. The probandwise concordance rates for monozygotic and dizygotic twins, the recurrence risk for ordinary siblings and the population prevalence are illustrated in Figure 2.

In Table 4, we report the tetrachoric correlations and variance component analyses for cleft lip with or without cleft palate and cleft palate for all twin pairs from the 1936 to 2004 cohorts. The highest tetrachoric correlation was found for monozygotic twins for both phenotypes when compared to dizygotic twins. Our data for both cleft lip with or without cleft palate and cleft palate suggested that the best fitting model was the AE model (lowest AIC). For cleft lip with or without cleft palate and cleft palate the heritability estimates (a^2) were very similar, 91% and 90% respectively, and the unique environmental factor (e^2) was small; 9% and 10% for cleft lip with or without cleft palate and cleft palate respectively. The estimates did not take difference in sex into account due to lack of sample size. When restricting attention to same sex dizygotic twins, the results did not change considerably and when we included the syndromic forms of oral cleft in the analyses, the estimates of heritability increased slightly (results not shown).

DISCUSSION

We found no excess risk of oral cleft for twins compared to singletons in this nationwide population-based study on a twin sample three times the size of the previously exploited

Danish twin sample. For the youngest cohorts, the oral cleft risk for twins was slightly increased, but the risk could not be distinguished from the singleton risk (Figure 1). This upward nudge in the oral cleft prevalence for twins compared to singletons for the youngest cohorts was likely due to a decrease in the infant mortality over time for twins in general and oral cleft twins in particular. Nor could we demonstrate an excess risk of oral cleft for the monozygotic twins when compared to singletons. We found the highest concordance for the monozygotic twins, along with the very high heritability estimates provides further support for a substantial genetic contribution to oral cleft etiology. However, a new indication of dizygotic twins having an excess risk of oral cleft compared to ordinary siblings justifies the continuing search for environmental factors of importance for the oral cleft etiology.

Strength and weaknesses. This was a large nationwide study based on a nearly complete population of isolated oral cleft individuals collected over 69 years. The ascertainment of both oral cleft cases and twins was high. However, some analyses were made for the 1968 to 2004 cohorts benefitting from the fact that the ascertainment for that period was close to complete with available zygosity for 80%. This study provided highly reliable results due to a threefold increased sample when compared to previous estimates on the Danish 1970-1990 cohorts (207 twins vs. the previous 65) [4,5]. Even when restricting analysis to the youngest cohorts, the twin sample was still twice the size (136 vs. 65) of the previous sample.

A decrease in neonatal mortality rates over time might have introduced a selection bias into the Danish Facial Cleft Database for the earliest cohorts. Before 1954, when midwifes in Denmark became obliged to report any oral cleft identified at birth to the National Institute of Defect of Speech, the oral cleft individuals had to survive until the age of 2 months to be evaluated for surgery and thereby be included in the Database.[26] The individuals with a cleft palate were most susceptible to this selection bias because they often entered the database later in life.[26] The late

entry was due to cleft palate individuals being 2 years old at surgery and the ones with the mildest

cleft palates were not in need of surgery at all. Moreover, the cleft palate individuals with an

associated syndrome or other anomalies may have had an even higher infant mortality since cleft

palate individuals were twice as likely to have an associated syndrome or other anomalies as the

cleft lip/cleft lip with cleft palates.[24] For the oral cleft twins, this problem might have been

magnified since at least one third of all the twins were born preterm with an accompanying higher

infant mortality. After 1954, all oral clefts also registered later in a child's life were reported to the

institutes which, along with the improvement of the neonatal care from the 1960s, have improved

the ascertainment markedly. This survival/selection bias was evident from our results on the cohorts

from 1936 to 1967 where there was a marked underrepresentation of twins with cleft palate

compared to singletons. We do not find it plausible that the smaller number of cleft palates was due

to a difference in diagnosing of cleft palate or oral cleft for twins relative to singletons. The total

twin population when drawn from The Danish Twins Registry also suffers from survival bias since

the twins in the oldest cohorts had to survive until the age of 6 to be included in the Registry.[25] For

the 1968 cohorts and onwards, 99% of all live born twins had been ascertained.

It has previously been shown that the zygosity determination in the Danish Twin

Registry has a high degree of validity.[27] However, the use of questionnaires regarding physical

resemblance might not be the best method in a study of facial malformation. From studies on two

subsets of our Danish oral cleft twin population from 1941-1969[28] and 1970-1990 [4,5] it was evident

that the method resulted in less than 5% misclassification of monozygotic twins as dizygotic twins.

Both studies used blood, serum, and enzyme determinants to verify the information obtained in the

questionnaire. In our study, the difference between the oral cleft probandwise concordance rate for

all dizygotic twins relative to the dizygotic same sex twins could indicate such bias. For the cleft lip

with or without cleft palates, however, no such difference could be found and since the cleft lip with

or without cleft palate individuals would be the most prone to misclassification due to the major facial asymmetry, any information bias introduced on this behalf was likely to be minimal. This assumption was supported by the similar monozygotic:dizygotic twin proportion for the cleft twin population and the total twin population and comparable proportions of monozygotic and dizygotic twins among the cleft lip, cleft lip with cleft palate, or cleft palate individuals.

The power issue continues to be a limiting factor when studying a relatively rare event such as oral cleft in twins. Over the period observed, the average twin frequency was 1.3%. With an oral cleft prevalence of 0.17% for the same period the probability of twinning to co-occur was one in 45,000 individuals.

We found no interaction between sex and zygosity that justified our subgroup analyses. The subgroup analysis, however, prompted multiple comparisons hence it cannot be ruled out that our results were chance findings. Caution should also be taken when making conclusions on differences found in subgroup analyses when no overall difference in the oral cleft prevalence between twins and singletons were found.

Comparison with previously published studies. Our study population is an expansion of the data exploited by Shields et al.[28] in 1979 and Christensen and Fogh-Andersen[4,5] in 1993. Our results benefitted from the three times larger twin sample for the 1936-2004 cohorts and two times larger for the youngest unselected 1968-2004 cohorts. The oral cleft occurrence among twins and singletons has also been studied on other populations, and on the largest studies probandwise concordance rates and heritability were estimated. Some studies were too small to draw anything else but a hypothesis.[9,12] Others were large but susceptible to either ascertainment bias,[14,34] inclusion of syndromic forms of oral cleft,[5,18,19,34] or without stratification for zygosity.[10,11,17] The majority of all these studies found no difference in the oral cleft prevalence for twins relative to singletons and these studies were based on a total number of twins more than twice the size of the

twin sample used in the studies suggesting a difference.[4,5,11,13,15,17,19-22,28,35] The largest of the previous studies also estimated pairwise or probandwise concordance rates,[4,5,19,22,28,35,36] only one recent study estimated heritability (cleft lip with or without cleft palate: a^2=0.73; standard error 0.42 and a^2=0.66; standard error 0.39, male and female respectively).[4]

The size and the quality of our Danish twin sample overcame most of the challenges mentioned and provided valid estimates of oral cleft occurrence for twins and singletons, probandwise concordance rates and heritability. We were able to explain that the decreased risk for twins found by Shields et al. on a subset of the Danish populations was likely due to survival bias. The excess oral cleft concordance for monozygotic twins compared to dizygotic twins support the large genetic component to the etiology of oral cleft, and our demonstration of a more than fourfold increased probandwise concordance rate for monozygotic twins relative to dizygotic twins agrees with several loci having an effect on the oral cleft etiology.[2,37] Nevertheless, less than 100% phenotypic concordance indicates that environmental factors could be of importance since the genomic sequence alone cannot explain the disease susceptibility. The effect of the environment is supported by the excess oral cleft risk for dizygotic twins relative to singleton siblings, which could be demonstrated for the first time in this study. This difference justifies the continued search for environmental factors of importance for the cleft etiology to help prevent oral cleft in the future. However, environmental effects may not sufficiently explain the monozygotic twin discordance, which could also result form genetic, cytogenetic or epigenetic anomalies in the affected twin, and not the other.[38-40]

A measure of the magnitude of the genetic and environmental effect was provided by use of a variance component analyses. We found that more than 90% of the variation in liability to oral cleft could be explained by genetic effects for both cleft lip with or without cleft palate and cleft palate. The best fitted model in the variance component analysis was the AE model suggesting

that the proportion of variance in the oral cleft occurrence is solely due to additive genetic factors (A) and unique (non-shared) environment (E).

      In conclusion, we found no evidence for a special etiology to oral clefting for twins although some studies have indicated that twins have an excess risk of oral cleft relative to singletons. We provided support for a substantial effect of genetic factors to both the cleft lip with or without cleft palate and cleft palate etiology. Moreover, for the first time an indication of an excess risk of oral cleft for dizygotic twins compared to singleton siblings could be demonstrated.

Contributors: All authors made substantial contributions to the intellectual content of the manuscript and all have approved the final version. DG had the primary responsibility for writing the paper and analyzing the data. KC initiated the study, obtained the funding, and supervised the analyses and the writing of the manuscript. JM and CB helped develop the protocol, provided constructive insights to the interpretation of the data and helped write the manuscript. JH, JK, and IP assisted the analysis and the writing of the manuscript. IP did the Mx analysis. AS provided the data material. DG is the guarantor.

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TABLE 1. Number and prevalence of twins and singletons (N=4,908,226) with isolated Oral Cleft, stratified for phenotype and sex; Denmark 1936-2004.

		Twins					Singletons				
Cohort	Phenotype/Source	Number	%	Prevalence per 10,000† (95% confidence interval)			Number	%	Prevalence per 10,000 † (95% confidence interval)		
		All	Male	Male	Female	All	All	Male	Male	Female	All
1936 – 2004											
	Affected										
	Cleft lip	72	75.0	8.1 (6.1-10.5)	2.8 (1.7-4.5)	5.5 (4.3-6.9)	2,495	63.9	6.5 (6.2-6.8)	3.9 (3.6-4.1)	5.2 (5.0-5.4)
	Cleft lip and palate	93	74.2	10.3 (8.0-13.1)*	3.8 (2.4-5.6)	7.1 (5.7-8.7)	3,039	69.7	8.6 (8.2-9.0)*	4.0 (3.7-4.2)	6.3 (6.1-6.6)
	Cleft palate	42	33.3	2.1 (1.1-3.5)*	4.4 (2.9-6.3)*	3.2 (2.3-4.3)*	2,432	45.1	4.5 (4.2-4.7)*	5.7 (5.4-6.0)*	5.1 (4.9-5.3)*
	Oral Cleft	207	66.2	20.5 (17.2-24.2)	11(8.6-13.9)	15.8 (13.8-18.1)	7,966	60.4	19.5 (19.0-20.1)	13.5 (13.1-14.0)	16.6 (16.2-17.0)
	Total Danish Population										
	DTR•	110,556	52.3				-	-			
	DST^	130,710	51.2				4,798,526	51.4			
1936 – 1967											
	Affected										
	Cleft lip	32	78.1	7.2 (4.7-10.7)	2.1 (0.9-4.4)	4.7 (3.2-6.7)	1,131	64.5	5.7 (5.3-6.1)	3.3 (3.0-3.7)	4.6 (4.3-4.8)
	Cleft lip and palate	30	70.0	6.1 (3.8-9.3)	2.7 (1.2-5.2)	4.4 (3.0-6.3)	1,401	70.7	7.8 (7.3-8.3)	3.4 (3.1-3.8)	5.6 (5.4-6.0)
	Cleft palate	9	44.4	1.2 (0.3-3.0)*	1.5 (0.5-3.5)*	1.3 (0.6-2.5)*	1,027	42.3	3.4 (3.1-3.7)*	4.9 (4.5-5.3)*	4.1 (3.9-4.4)*
	Oral Cleft	71	70.4	14.4 (10.7-19.0)	6.3 (3.9-9.7)	10.5 (8.2-13.2)*	3,559	60.5	16.9 (16.2-17.6)	11.7 (11.1-12.3)	14.3 (13.9-14.8)*
	Total Danish Population										
	DTR•	48,142	53.6				-	-			
	DST^	67,746	51.1				2,482,528	51.4			
1968 – 2004											
	Affected										
	Cleft lip	40	72.5	9 (6.0-12.9)	3.6 (1.8-6.4)	6.4 (4.5-8.7)	1,364	63.4	7.3 (6.8-7.8)	4.4 (4.1-4.8)	5.9 (5.6-6.2)
	Cleft lip and palate	63	76.2	14.9 (11.0-19.7)*	4.9 (2.7-8.1)	10 (7.7-12.8)*	1,638	68.9	9.5 (8.9-10.1)*	4.5 (4.1-4.9)	7.1 (6.7-7.4)*
	Cleft palate	33	30.3	3.1 (1.5-5.7)	7.5 (4.8-11.2)	5.2 (3.6-7.4)	1,405	47.3	5.6 (5.2-6.0)	6.6 (6.1-7.1)	6.1 (5.8-6.4)
	Oral Cleft	136	64.0	27 (21.6-33.3)	16 (11.8-21.1)	21.6 (18.1-25.6)	4,407	60.3	22.3 (21.5-23.2)	15.5 (14.8-16.3)	19 (18.5-19.6)
	Total Danish Population										
	DTR•	62,414	51.2				-	-			
	DST^	62,964	51.2				2,315,998	51.4			

Abbreviations: DTR, Danish Twin Registry; DST, Statistics Denmark
•Danish Twin Registry; ^Statistics Denmark; †livebirths, all prevalence estimates use DST reference
*Significant differences between twins and singletons with p < 0.05 using Poisson regression, controlling for cluster

TABLE 2. Comparison of twins and singletons according to oral cleft phenotype, sex and zygosity; Denmark 1968-2004.

Cohort	Phenotype/Source	All	Male	Prevalence Ratio between Twins and Singletons (95% CI)		
		N	%	Male	Female	All
All OC Twins†						
	Cleft lip	40	72.5	1.25 (0.82-1.89)	0.82 (0.45-1.48)	1.09 (0.77-1.53)
	Cleft lip and palate	63	76.2	1.58 (1.16-2.16)*	1.09 (0.65-1.82)	1.43 (1.09-1.9)*
	Cleft palate	33	30.3	0.56 (0.30-1.05)	1.15 (0.75-1.77)	0.87 (0.61-1.26)
	Oral Cleft	136	64.0	1.22 (0.96-1.54)	1.04 (0.77-1.40)	1.15 (0.95-1.38)
MZ Twins†						
	Cleft lip	9	55.6	1.57 (0.55-4.43)	1.98 (0.74-5.3)	1.71 (0.83-3.52)
	Cleft lip and palate	9	77.8	1.68 (0.66-4.26)	0.97 (0.24-3.89)	1.42 (0.65-3.12)
	Cleft palate	4	25.0	0.41 (0.10-2.90)	1.0 (0.23-4.32)	0.74 (0.22-2.45)
	Oral Cleft	22	59.1	1.33 (0.66-2.65)	1.27 (0.58-2.79)	1.29 (0.77-2.17)
DZ Twins†						
	Cleft lip	17	64.7	0.85 (0.45-1.63)	0.78 (0.35-1.75)	0.82 (0.50-1.37)
	Cleft lip and palate	39	76.9	1.78 (1.23-2.59)*	1.15 (0.59-2.22)	1.57 (1.13-2.20)*
	Cleft palate	20	25	0.51 (0.21-1.22)	1.32 (0.79-2.20)	0.94 (0.59-1.49)
	Oral Cleft	76	60.5	1.16 (0.86-1.57)	1.12 (0.78-1.60)	1.14 (0.90-1.45)

Abbreviations: DTR, Danish Twin Registry; DZ, dizygotic; MZ, monozygotic; DST, Statistics Denmark
*Significant differences between twins and singletons with $p < 0.05$
†Controlled for cluster

FIGURE 1. Prevalence Ratio for Oral Cleft Twins vs. Singletons
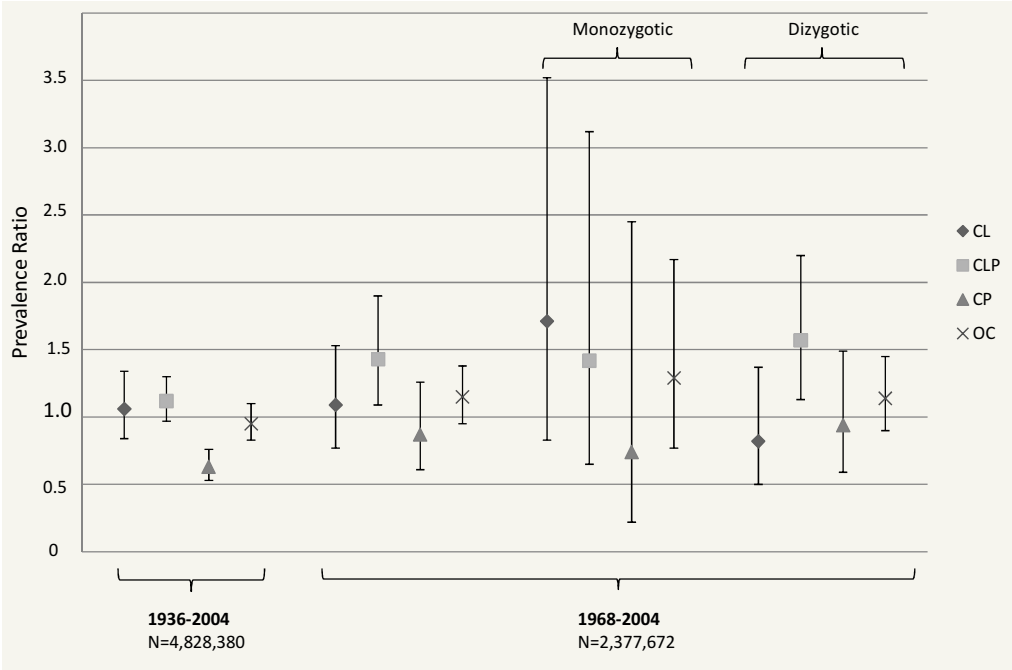
Abbreviations: CP, cleft palate; CL, cleft lip; CLP, cleft lip with cleft palate; OC, all oral clefts

TABLE 3. Probandwise concordance rates for twins and recurrence risk for siblings for isolated OC, Denmark 1936-2004 (N=185 twin pairs/7,654 sib pairs)

Phenotype	MZ		DZ all		DZ same sex		UZ		Siblings		p value for comparison[^]	
Concordance	N	C_{Pr},% (95% CI^)	N	C_{Pr},% (95% CI^)	N	C_{Pr},% (95% CI^)	N	C_{Pr},% (95% CI^)	N	Recurrence risk*, % (95% CI^)	MZ vs. DZ all[$]	DZ all vs. siblings
Cleft lip with or without cleft palate												
Concordant pairs	8	50 (32-68)	4	7.9 (3.5-15)	2	7.7 (2.1-19)	5	31 (16-50)	86	3.2 (2.7-3.7)	<0.0001	0.02
Discordant pairs	16		93		48		22		5224			
Cleft palate												
Concordant pairs	1	33 (4.3-78)	1	7.4 (0.91-24)	0	0	0	0	36	3.0 (2.3-3.8)	0.15	0.20
Discordant pairs	4		25		17		6		2308			
Oral Cleft												
Concordant pairs	9	47 (31-64)	5	7.8 (3.8-14)	2	5.6 (1.6-14)	5	26 (13-43)	122	3.1 (2.8-3.5)	<0.0001	<0.01
Discordant pairs	20		118		67		28		7532			

*Recurrence risk from 1952 to 2005
^Exact methods for 95% confidence intervals (CI) and significance testing, significance level < 0.05
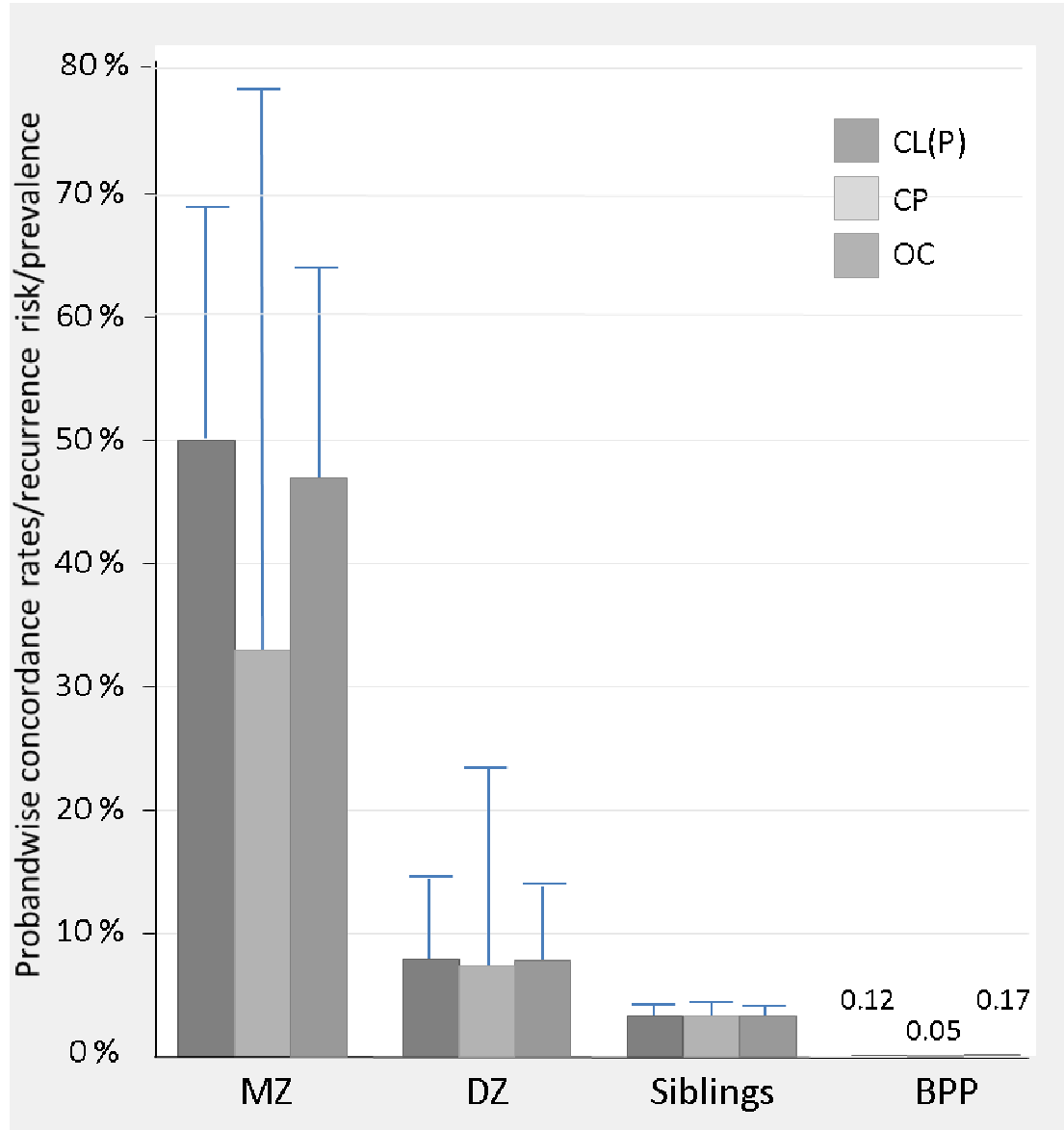$Under the assumption of equal prevalence for MZ and DZ twins

TABLE 4. Tetrachoric correlations and variance component analyses for Danish twins with isolated Oral Cleft, 1936-2004 (N=185)

N	Cleft lip with or without cleft palate		Cleft palate	
MZ pairs[*]	8/16		1/4	
DZ pairs[*]	4/93		1/25	
Zygosity	**r (95% CI)**	**p Value**	**r (95% CI)**	**p Value**
MZ	0.91 (0.79 - 0.97)	<0.001	0.88 (0.47 - 0.99)	0.20
DZ	0.55 (0.35 - 0.70)		0.60 (0.29 - 0.83)	

Model Fit Statistics

	vs.	x^2	d.f.	AIC	p Value	x^2	d.f.	AIC	p Value
ACE	SAT	0.78	3	-5.23	0.86	0.12	3	-5.88	0.99
ADE	SAT	1.76	3	-4.24	0.62	0.94	3	-5.07	0.82
AE	*ACE*	*0.99*	*1*	*-6.24*	*0.32*	*0.82*	*1*	*-7.07*	*0.37*
CE	ACE	13.60	1	6.37	<0.001	1.64	1	-6.24	0.20
E	AE	107.97	1	99.73	<0.001	20.24	1	11.36	<0.001

Heritability estimates (95%CI)	a^2	e^2	a^2	e^2
	0.91 (0.82 - 0.97)	0.09 (0.03 - 0.18)	0.90 (0.60 - 0.99)	0.10 (0.01 - 0.40)

Abbreviations: A, additive genetic factors; AIC, Akaike's Information Criterion; a^2 and e^2, standardized parameter estimates (95% CI); C, common environmental factors; CI, confidence interval; d.f., degrees of freedom; DZ, dizygotic; D, dominant genetic factors; E, non-shared environmental factors; MZ, monozygotic; SAT, saturated model; r, tetrachoric correlations
[*]Complete pairs (i.e. both twins have a score)/broken pairs (i.e. only one twin has a score)
Note: Best fitting model in *italic*

FIGURE 2. Probandwise concordance rates for mono- and dizygotic twins, recurrence risk for ordinary siblings, and population prevalence for CL(P) and CP

Abbreviations: BPP, Background Population Prevalence; CL(P), Cleft lip with or without cleft palate; CP, cleft palate; DZ, dizygotic; MZ, monozygotic; OC, oral clefts