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A Preliminary Investigation into Orofacial Clefts in the Central and Western Regions of Saudi Arabia

Sabbagh, Heba J.

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A PRELIMINARY INVESTIGATION INTO OROFACIAL CLEFTS IN THE CENTRAL AND WESTERN REGION OF SAUDI ARABIA

A PRELIMINARY INVESTIGATION INTO OROFACIAL CLEFTS IN THE CENTRAL AND WESTERN REGIONS OF SAUDI ARABIA

Heba J Sabbagh

BDS, MSc

Thesis submitted for the degree of Doctor of Philosophy

The Faculty of Medicine, Dentistry and Nursing,

University of Dundee.

Division of Oral Sciences

Dundee Dental Hospital and School

University of Dundee

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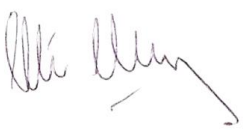
Declaration

I declare that the work presented in this thesis is all my own work, has not previously been accepted for a higher degree and I have consulted all references cited.

Heba Sabbagh

I confirm that the conditions of the relevant Ordinances and Regulations of The University of Dundee have been fulfilled.



Professor P. A. Mossey (Supervisor)

University of Dundee

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Abstract

Background and aims: Clefts of the lip and palate are the most common types of craniofacial birth defects found worldwide. This study is the first multicentre case-control triad (of children and their parents) in Saudi Arabia that aims to: (I) measure the prevalence of non-syndromic orofacial cleft (NSOFC) at birth and (II) investigate the genetic and environmental risk factors associated with NSOFC in infants attending government hospitals in the Western and Central Regions of Saudi Arabia.

Material and methods: Two hundred and seventeen non-syndromic orofacial cleft (NSOFC) triads comprising probands aged 18 months or less were selected from eleven hospitals in three main cities of Saudi Arabia (Jeddah, Maddina, and Riyadh). Patients were examined to identify cleft phenotype according to the LASHAL classification. Cases born in the designated hospitals from January 2010 to January 2012 were compared with the total number of births, in the same period of time to measure the prevalence of CL/P and CP in Saudi Arabia. Cases were compared with 244 control triads matched for proband age, gender and location to assess the environmental and genetic (IRF6 and VAX1 genes) aetiology of NSOFC through a questionnaire and infant-parental triad saliva sample. Gene-environmental interaction (GEI) was assessed through measuring the distribution of maternal genotypes and alleles according to exposure/no-exposure environmental factors.

Results: The prevalence of NSOFC in government hospitals in the Western and Central regions of Saudi Arabia was 1.17/1000 births.

Environmental risk factors (ERFs) significantly related to NSOFC after the odds ratios were adjusted through logistic regression included; family history for NSOFC,

folic acid, antibiotic use, common cold/flu, maternal stress, paternal waterpipe smoking, incense exposure, maternal exposure to chemicals and maternal main water source.

Genetic analysis revealed significant over-transmission of the common *IRF6* allele rs2013162 in CL/P families using Family based association test (FBAT) analysis and paternal transmission using PLINK testing. Two haplotypes containing the rare alleles of *VAX1* rs4752028 and *IRF6* rs2013162 were significantly associated with NSOFC. *VAX1* showed significant difference between cases and controls infant parental triad.

Gene-environment interaction (GEI) found a significant relationship between maternal SNP (IRF6 rs2013162 and/or VAX1 rs7078160) and maternal folic acid pre-gestation ingestion, antipyretic medication ingestion, fever, abdominal pain, high blood pressure, passive smoking, maternal stress and paternal waterpipe smoking, and /or maternal passive smoking

Conclusion:

- The prevalence of NSOFC in Saudi Arabia (1.17/1000 births) is marginally lower than global average figures (1.25/1000births)
- Maternal exposure to common cold/flu, folic acid supplementation, stress, antibiotic use, incense, source of drinking water, paternal waterpipe smoking and intense paternal tobacco smoking are associated with increased risk of CL/P and/or CP in Saudi Arabia.
- *IRF6* rs2013162 showed significant over transmission of the common allele (C) with CL/P cases. Also, *VAX1* rs4752028 and rs7078160 rare allele are

found more frequent in CL/P and CP infant-parental triad cases compared to controls except for paternal rs7078160 rare homozygous allele.

- This study gives a preliminary suggestion of GEI and is considered a valuable instrument for public health strategies

Summary

Background and aims: Clefts of the lip and palate are the most common types of major craniofacial birth defects and are among the most frequent congenital anomalies found worldwide. Affected individuals have a range of functional as well as aesthetic problems from birth through adulthood requiring a lifelong series of interventions. This study is the first multicentre case-control triad (of children and their parents) in Saudi Arabia it aims to: (I) measure the prevalence of non-syndromic orofacial cleft (NSOFC) at birth and (II) investigate the genetic and environmental risk factors associated with NSOFC in infants attending government hospitals in the Western and Central regions of Saudi Arabia.

Material and methods: Two hundred and seventeen non-syndromic oral cleft (NSOFC) triads comprising probands aged 18 months or less were compared with 244 control triads matched for proband age, gender and location, were selected from eleven hospitals in three main cities of Saudi Arabia (Jeddah, Maddina, and Riyadh). Patients were examined to identify the cleft phenotype according to the LASHAL classification.

Part I. Prevalence of oral cleft in Saudi Arabia:

Part I of this study included all cases born in the designated hospitals from January 2010 to January 2012. Infants born with NSOFC were compared with the total number of births, in the same period of time.

Part II. Aetiology of oral clefts:

Environmental risk factors:

Part II of our study, data were collected on environmental risk factors using a modified WHO questionnaire, covering events in the three-month pre-gestation through to the first trimester period.

Genetic risk factors:

To identify genetic etiological risk factors, DNA was extracted from infants' and parents' saliva samples obtained using Oragene sample collection kits. Single nucleotide polymorphisms (SNPs) at two candidate gene loci: *Interferon regulatory factor 6* (*IRF6*) (rs2013162, rs2235375 and rs2235371) and *Ventral anterior homeobox 1* (*VAX1*) (rs7078160 and rs4752028) were genotyped using restriction digestion PCR (for *IRF6*) and real time PCR (for VAX1) methodologies.

Gene-Environmental Interaction (GEI):

GEI was assessed through measuring the distribution of maternal genotypes and alleles according to exposure/no-exposure to environmental factors. Two types of study designs are carried out to assess gene-environmental interaction; case-only and case-control study designs.

Results: for the study measured the prevalence and aetiology of NSOFC in Saudi.

Part I: The prevalence of NSOFC:

In government hospitals in the Western and Central regions of Saudi Arabia was 1.17/1000 births.

Part II: aetiology of NSOFC:

Environmental risk factors (ERFs) significantly related to NSOFC after the odds ratios were adjusted through logistic regression were:

- For cleft lip (CL), the predictor variables were: family history for NSOFC (OR:8.79, 95%CI:3.92to19.68); antibiotic ingestion during the pregestation (OR:3.01, 95%CI:1.1to8.61) and during the 1st trimester period (OR:3.07, 95%CI:1.4to6.73); mothers complaining of family problems (OR:2.29, 95%CI:1.21to4.35); and incense exposure in the 1st trimester period (OR:0.51, 95%CI:0.27to0.94).

- For cleft lip and palate (CLP) the associated variables were: family history for NSOFC (OR:14.73, 95%CI:5.99to36.17); common cold/flu during the pre-gestation period (OR:5.82, 95%CI:2.38to14.25); folic acid in the 1st trimester (OR: 0.09, 95%CI:0.14to0.51) maternal stress (OR:3, 95%CI:1.49to6.03); paternal waterpipe smoking (OR:5.74, 95%CI:2.07to15.9); maternal exposure to chemicals in the pregestation period (OR:2.91, 95%CI:1.44to5.89) and maternal main water source (P= 0.001). Maternal Zamzam drinking water shows a reduced chance of having an infant with CLP compared to well water (OR:0.02, 95%CI:0.002to0.2),

- For cleft palate (CP), the factors were: family history (OR:5.89, CI:2.36to14.74); maternal common cold/flu in the pregestation period (OR:2.28, 95%CI:1.09 to4.77); abdominal pain in the 1st trimester (OR:5.81, 95%CI:2.05to16.45); and maternal stress (OR:2.1, 95%CI:1.08to4.06).

Genetic risk factors:

Hardy Weinberg equilibrium (HWE) testing showed no significant differences between the expected and observed allele frequencies at one of the tested loci (VAX1 rs4752028). Family based association test (FBAT) analysis and PLINK testing found a significant over-transmission of the common *IRF6* allele rs2013162 in NSOFC families (P= 0.014 and P= 0.016, respectively) and cleft lip with or without cleft palate (CL/P) families (P= 0.018 and 0.015 respectively), with paternal transmission of the significant variable (P= 0.05). Two haplotypes containing the rare alleles of *VAX1* rs4752028 and *IRF6* rs2013162 were significantly associated with NSOFC (P= 0.021 and P= 0.01).

A paternal *VAX1* rs4752028 homozygous common allele genotype (TT) was identified significantly more often in controls versus cases and the paternal heterozygous genotype (CT) identified to be significantly more frequent in cases versus controls across the different cleft phenotypes. The homozygous rare allele genotype (CC) was found significantly more often in both infants and mothers in case versus in control triads for the NSOFC and CL/P phenotypes; the homozygous common allele genotype (TT) occurred significantly more often in control triads for NSOFC and CL/P categories; the heterozygous genotype (CT) was present significantly more often in NSOFC and its sub-phenotype triad individuals except in mothers of probands with CP. The rare C allele was identified significantly more often in case versus control triad individuals and was associated with: NSOFC (fathers: OR:2.24, 95%CI:1.47to3.4; mothers: OR:2.44, 95%CI:1.61to3.7; and infants: OR:2.71, 95%CI:1.78to4.13); CL/P (fathers: OR:2.16 and95%CI:1.38to3.4; mothers: OR:2.39, 95% CI:1.53to3.71; and infants with OR:2.77, 95%CI:1.77to4.34);

and CP (fathers: OR:2.24 and 95% CI:1.15to4.36; mothers: OR:1.97, 95%CI:0.99to3.93; and infants: OR:2.43, 95%CI:1.25to4.7).

For *VAX1* rs7078160, the homozygous rare allele genotype (AA) was significantly present more often in NSOFC and CL/P infants compared to controls. The homozygous common allele genotype (GG) was significantly present more often in infant-parental control triad individuals than in NSOFC case triad individuals ($P< 0.05$). The heterozygous genotype (AG) was significantly more prevalent in parental controls compared to parents of NSOFC and CL/P patients ($P< 0.05$). There were statistically significant differences between case and control triad individuals for CL/P (fathers: OR: 1.73 and 95% CI: 1.05 to 2.86, mothers: OR: 2.43 and 95% CI: 1.49 to 3.97; and infants: OR: 2.34 and 95% CI: 1.44 to 3.81) with significantly greater frequency of the rare allele in case compared to control triad individuals.

Analysis of parental consanguinity status versus infants' genotype identified a statistically significant increase in NSOFC infants carrying the *VAX1* rs4752028 rare C allele compared to control infants: for NSOFC, OR: 3 and 95% CI: 1.55 to 5.4; for CL/P, OR: 2.97 and 95% CI: 1.54 to 5.76; and for CP, OR: 6.52 and CI: 3.1 to 13.7.

Gene-environment interaction (GEI):

Multi-nominal logistic regression analysis for significant gene-environmental interaction among cases found; for maternal rs2013162, homozygous rare allele genotype (AA) was significantly related to antipyretic medication in the 1st trimester period (OR:10.18, 95%CI:1.31to79.1) and abdominal pain in the 1st trimester period (OR:7.4, 95%CI:1.2to45.51) among NSOFC cases. Heterozygous allele genotype

(AC) was significantly related to folic acid pre-gestation (OR:6.78, 95%CI:1.41to33.49) and fever pre-gestation (OR:0.23, 95%CI:0.06to0.83) among NSOFC cases.

For maternal VAX1 rs7078160, homozygous rare allele genotype was significantly related to paternal waterpipe (OR:6.95, 95% CI:1.5to32.2) and maternal exposure to high blood pressure in the 1st trimester period (OR:11.2, 95%CI:1to125.73) among NSOFC; For *IRF6* rs2013162, a statistically significant increase in the frequency of the homozygous rare allele genotype (AA) was seen in mothers from control versus NSOFC triads in those mothers who ingested folic acid supplementation in the 1st trimester (P= 0.003). In contrast, there was a statistically significant increase in the frequency of the rare (AA) genotype in NSOFC versus control mothers positive for maternal passive smoking (P= 0.006); maternal stress (mothers complaining of family problems (P= 0.003), of being under stress (P= 0.015), and of suffering abdominal pain in the 1st trimester (P= 0.007)); and maternal exposure to chemicals in the pre-gestation and 1st trimester period.

for case-control study design; stratification of *IRF6* rs2235375 genotypes in mothers according to different environmental factors showed a statistically significant elevation in the frequency of the maternal rare allele homozygous genotype (GG) in case versus control mothers reporting maternal flu/common cold in the pre-gestation period (P< 0.001) and fever in the pre-gestation period (P= 0.007). Maternal *VAX1* rs4752028 GEI analysis showed a statistically significant increase in the homozygous common allele genotype (TT) in control versus case mothers positive for maternal folic acid supplementation in the 1st trimester period (0.023); maternal multivitamin supplementation in the pre-gestation period (0.026); and maternal intake of Zamzam water (0.001). There was also a significantly higher frequency of the heterozygous

genotype (CT) in NSOFC maternal controls versus cases reporting calcium supplementation in the 1st trimester period (0.025). *VAX1* rs7078160 genotype analysis demonstrated a statistically significant increase in the frequency of NSOFC mothers carrying the homozygous rare allele genotype (AA) compared to controls mothers, in triads with identified parental consanguinity (0.049). Genotype-environment interaction analysis demonstrated that there were a significantly greater number of controls carrying the maternal homozygous common allele genotype (GG) than NSOFC mothers with a history of maternal folic acid supplementation in the 1st trimester (0.029) and of drinking Zamzam water (0.001).

Conclusion:

- The prevalence of NSOFC in Saudi Arabia (1.17/1000 births) is marginally lower than global average figures (1.25/1000births) with CL/P is 0.89/1000 births and CP (0.28/1000 births). The prevalence of consanguinity in NSOFC infants is 81 (65.9%) cases.

- Maternal exposure to common cold/flu, folic acid supplementation, stress, antibiotic use, incense, source of drinking water, intense paternal tobacco smoking and paternal waterpipe smoking are associated with increased risk of CL/P and/or CP in Saudi Arabia.

- TDT and PLINK analysis is significant only for *IRF6* rs2013162 SNP which showed significant transmission of the common allele (C) with CL/P cases. Parent of origin is showed significant over transmission of the IRF6 variant from the paternal side in CL/P cases. CL/P and controls for maternal *IRF6* rs2013162 variant showed a significantly more homozygous rare allele

genotype (AA) in controls and more heterozygous allele genotype (CA) in CL/P cases.

- *VAX1* rs4752028 and rs7078160 rare homozygous allele and rare allele are found more frequent in CL/P and CP infant-parental triad cases compared to controls except for paternal rs7078160 rare homozygous allele.

- Two haplotype blocks, consisting of the five SNPs included in this study, showed a significant association with NSOFC.

- Maternal exposure to antipyretic, folic acid, fever, antibiotics, illnesses, common cold/flu, paternal waterpipe smoking, stress and chemicals can significantly interact with mothers *IRF6* (rs2013162 and rs2235375) gene variants affecting the risk of having a child with oral cleft.

- Maternal usage of folic acid and antibiotics; exposure to fever, illness, stress, high blood pressure and waterpipe smoking can significantly interact with mothers *VAX1* (rs4752028) and/or *VAX1* (rs7078160) gene variant affecting the risk of having a child with oral cleft.

- This study is considered a valuable instrument for public health strategies as it gives preliminary description of GEI for two genes (VAX1 and IRF6) in Saudi Arabia. It also directs future research in generating hypothesis to be tested for confirmation in studies with adequate sample power.

Ethical Approval

All the collaborating centres obtained ethical approval from the ethics committees of the Ministry of Health in Saudi Arabia and various hospitals (King Fahad Medical Ci National Guard Health Affair, Riyadh Military Hospital, King Abdulaziz Medical City, King Abdulaziz University Hospital, and King Fahad Armed Forces Hospital). The parents of cases and controls gave informed consent for participation in the case-control/case-triads study.

Dedication

To my father, mother, and husband who encouraged and supported me at every step to pursue this academic fulfilment. To Prof. P.A. Mossey, Dr. N. Innes, and Prof. N. Alamoudi who conceived the project, supervised its completion, and served as a mentor to me. To Prof Mona Hussain, Prof. Doaa EL Derwi, Dr Aziz Butali, and Dr. Sherif Edris who helped me to carry out the statistical analysis.

List of abbreviations

ABCA4	ATP-binding cassette transporter 4
BMP	Bone morphogenic proteins
BTEX	Benzene, toluene, ethyl benzene, and xylene
CL	Cleft lip only
CL/P	Cleft lip with or without cleft palate.
CLP	Cleft lip and palate
CP	Cleft palate only
DLX	Distal-less homeobox-containing
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
ERF	Environmental risk factors
FBAT	Family based association test
FDA	Food and Drug Administration
FGF	Fibroblast Growth Factors
FGFR	Fibroblast growth factor receptors
FOXE1	Fox head protein E1

GBD	Global Burden of Disease
GDP	Gross Domestic Product
GEI	Gene-environmental interaction
GWA	Genome-wide association studies
HAS1	Hyaluronan Synthase isozyme
HAPEM	Hazardous Air Pollutant Exposure Model
HCCSCA	Hungarian Case-Control Surveillance of Congenial Abnormality
HWE	Hardy Weinberg equilibrium
ICBDMS	International Clearinghouse for Birth Defects Monitoring System
IRF6	Interferon regulatory factor 6
IRB	Institutional Review Board
KAUH	King Abdulaziz University Hospital
KAMC	King Abdulaziz Medical City
KFH	King Fahad Hospital
KFAH	King Fahad Armed Hospital

KFMC	King Fahad Medical City
KFSHRC	King Faisal Specialized Hospital and Research Centre
MAFB	V-maf musculoaponeurotic fibrosarcoma oncogene homolog B
MEE	Medial edge epithelium
MOH	Ministry of Health
MSX1	Muscle specific homeobox 1
MTHFR	Methylenetetrahydrofolate
NSOFC	Non-syndromic orofacial cleft
NTD	Neural tube defect
OFC	Orofacial cleft
PCR	Polymerase chain reaction
PLINK	open-source whole genome association toolset
RNGH	Riyadh National Guard Hospital
RMH	Riyadh Military Hospital
PS	Palatal shelf

SES	Socioeconomic status
SHH	Sonic hedgehog
SNP	Single nucleotide polymorphism
SPSS	Statistical Package for the Social Sciences
SUMO	Small ubiquitin-like modifier
TBE	Tris/Borate/EDTA
TDT	Transmission disequilibrium test
TGFα	Transforming growth factor alpha
TGFβ	Transforming growth factor beta
TP63	Tumour protein 63
VAX1	Ventral anterior homeobox 1
WHO	World Health Organisation

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Chapter 1: Introduction

1.1 Introduction

Orofacial clefting (OFC) is a group of congenital anomalies comprised of cleft lip with or without cleft palate (CL/P) and isolated cleft palate (CP) (Mossey, 2001b). It may present as a part of a syndrome or associated with other abnormalities (Mossey et al., 2009). Non-syndromic orofacial cleft (NSOFC) has been described in the literature as OFC that either occurs in isolation, or is associated with one other major congenital abnormality or a few rare congenital abnormalities (Tolarova and Cervenka, 1998).

NSOFCs are the most common craniofacial defects globally, occurring in, on average, 1.25/1000 live births (Mossey and Modell, 2012b). OFC can lead to a series of functional as well as aesthetic problems including feeding difficulties (especially at birth), swallowing and nasal regurgitation, and hearing and speech difficulties. Although cleft defects can be surgically repaired in early childhood, residual deformities due to scarring and abnormal facial development, result in long-lasting functional and psychosocial problems (Broder et al., 1994). Affected individuals have higher morbidity and mortality throughout life than do unaffected people (Ngai et al., 2005). Therefore, clefts has adverse effects on the health and social integration of those affected (Christensen et al., 2004; Nopoulos et al., 2007).

In 2002, the WHO recommended including cleft lip and palate in their Global Burden of Disease (GBD). Although not actually included until 2010, this has stimulated interest among some member states to place greater emphasis on orofacial cleft treatment, epidemiology and research.

The aetiology of NSOFC is complex with many contributing factors: genetic, environmental and involve gene-environment interactions (Mossey et al., 2009). Understanding the

contribution of each to the aetiology of cleft lip and palate is complex. The number of genes involved, the differences between cleft lip and palate versus isolated cleft palate, the heterogeneity within each group, the type of inheritance patterns compounded by interaction with environmental factors, and the ethnic and geographic variation make it difficult to identify causal factors (Mossey and Little, 2009). Moreover, it has been reported in several studies that CL/P and isolated CP, with some exceptions, do not segregate in the same family (Jones, 1988; Dixon et al., 2011) nor occur as pairs in the same twins (Grosen et al. 2011), further suggesting separate genetic and biological aetiological variables. Current research, involving a range of different methodologies and strategies is ongoing in an attempt to identify the environmental and genetic factors involved. This research is the first multicentre study in Saudi Arabia to report the birth prevalence of NSOFC and investigate the genetic and environmental factors involved in the aetiology of NSOFC.

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Chapter 2: Literature Review

2.1 The Epidemiology of OFC

2.1.1 Global epidemiology of NSOFC

NSOFC comprises approximately 70% and 50% of OFC cases, as cited by Dixon et al. (2011) and FitzPatrick and Farrall (1993), respectively, and is the most common craniofacial defect found worldwide (Mossey and Castilla, 2001). The estimated overall global prevalence of NSOFC is 1.25 in every 1000 live births (Mossey and Modell, 2012b). However, birth prevalence varies considerably across geographic areas and ethnic groupings, for example, OFC occurs more commonly among Asian and Native American populations (about 2 in every 1000 births) than among Africans (about 1 in every 2500 births) (Mossey and Little, 2002). Despite efforts to record the frequency of birth defects over the years, accurate data on their epidemiology does not exist in many countries (Mossey and Castilla, 2001).

NSOFC prevalence also varies by gender. Over 50% of NSOFC cases generally occur in males. However, among the different sub-phenotypes of NSOFC, CL/P has a 2:1 male to female ratio whereas CP has a 1:2 male to female ratio. The most common NSOFC phenotype is cleft lip with cleft palate (CLP), followed by CL and finally CP. Clefting of the left side in CL/P is more common than the right side (2:1 ratio) (Dixon et al., 2011; Mossey and Modell, 2012b).

About one third of NSOFCs are associated with other birth defects. However, the reported prevalence of associated anomalies varies between 21% and 63.4% depending on diagnostic expertise, ascertainment, and definition of associated anomalies (Mossey, 2001b, Shprintzen et al., 1985). The most commonly reported associated anomaly is congenital heart disease (Milerad et al., 1997; Mossey, 2001b). Other associated anomalies that occur with NSOFC

include: anomalies of the head and neck as micrognathia; urogenital and renal anomalies; neural defects as neural tube defects; and limb defects. In addition, Rittler et al (2008) conducted a study to identify other anomalies in 1416 OFC cases, and found a positive correlation between anencephaly and CL/P (P< 0.001, OR: 3.4 and 95% CI: 2.2 to 5.3) and CP (P= 0.018, OR: 1.9, and 95% CI: 1.1 to 3.4). Furthermore, a significant relationship was identified between both club-foot and ear anomalies with NSOFC (Rittler et al., 2008).

2.1.2 The Epidemiology of OFC in Saudi Arabia

In Saudi Arabia, where almost 300,000 children are born per year (Ministry of Health, 2010), there is no national registry for OFC. One pioneer project that initiated registration of cleft lip and palate anomalies in Saudi Arabia was carried out in the King Faisal Specialised Hospital and Research Centre in Riyadh (the capital city of Saudi Arabia). There were 1555 patients with OFC and/or other craniofacial anomalies registered over a period between 1999 and 2008, of which 774 were OFC cases (Al-Johar et al., 2009). However, this registration was limited only to patients referred to the King Faisal Specialised Hospital and Research Centre which is not the only centre that manages OFC in Saudi Arabia.

In 2012, a systematic review of the prevalence of NSOFC in Saudi Arabia and other Middle Eastern countries found a large variation in reported NSOFC birth prevalences, ranging from 0.3 to 2.19 per 1000 births (Sabbagh et al. 2012)**.** The complete details of the systematic review are presented in Appendix B1.

2.2 OFC Syndromes

Syndromic OFC compromises almost 30% of OFC cases and frequently presents with CP

(almost 50% of cases) (Mossey and Castilla, 2001). Potential factors that could lead to syndromic OFC include: regional gene deletion in syndromic disorders such as 22q11 deletion; clinical sequences such as Pierre Robin; Mendelian disorders such as Van der Woude syndrome; or teratogenic effects such as seen in foetal alcohol syndrome (Cohen, 1978). A list of syndromes associated with OFC and their related genes are presented in Appendix A1.

2.2.1 Pierre Robin sequence

Pierre Robin sequence is a combination of features that can present together, alone or as part of a syndrome. These features include CP, micrognathia, and glossoptosis. The sequence of developmental defects is explained by the mechanical obstruction caused by a micrognathic mandible leading to glossoptosis and a retropositioning of the tongue that prevents palatal fusion and causes life threatening respiratory obstruction and feeding difficulties (Smith, 1975; Tan et al., 2013). The birth prevalence of the Pierre Robin sequence was reported to be 12.4 per 100,000 live births (Vatlach et al., 2014).

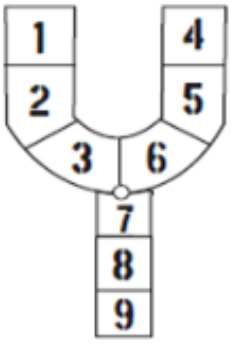
Printzlau and Andersen (2004) described the clinical presentation and epidemiology of Pierre Robin sequence retrospectively in the Danish population from 1990 to 1999. They concluded from their research that Pierre Robin sequence could be considered as a separate clinical entity with the main feature (in almost 60% of patients) being a U-shaped cleft palate. Almost 12% of cases had Stickler syndrome, a rare autosomal dominant connective tissue disorder that is associated with ocular abnormalities (Printzlau and Andersen, 2004; Pacella et al., 2010).

2.3 Classification of NSOFC

There are many classification systems proposed for OFC in the literature that aim to describe the morphology, extension and severity of oral cleft (Shah et al., 2012). This paper will present some of these classifications

2.3.1 Kernahan's striped Y (Figure 2.1)

Kernahan's striped Y is one of the most commonly used systems of OFC classification. It provides a graphic classification scheme using a symbolic letter Y. Each line of the Y configuration represents a fusion line between two orofacial segments. In order to show the clefting area, stippling of the corresponding fusion line is carried out to the degree of orofacial clefting that is accordingly divided into: lip clefting; alveolus clefting; premaxilla clefting; soft palate and hard palate clefting. Cross-hatching represents a sub-mucous CP.



(Kernahan, 1971)

Figure 2.1: Kernahan's striped Y classification

2.3.2 Modified Kernahan's striped Y

The standard Kernahan's striped Y classification was modified with the addition of two triangles in the upper end of the Y configuration. These triangles represent the fusion line between the maxillary prominence and medial nasal prominence. They illustrate nasal deformity and allow easy discrimination of Simonart's band. Blackening of the squares means that the alveolar segments are collapsed. Furthermore, an arrow was introduced to represent the degree of maxillary protrusion (Elsahy, 1973).

2.3.3 LAHSHAL classification

LAHSHAL classification subdivides the cleft lip and alveolus according to side (right or left), and palate (hard and soft) (Kriens, 1989). In 2005, the Royal College of Surgeons in Britain modified the classification by omitting one H from the classification acronym. The letters in the LASHAL abbreviation stand for: lip (L); alveolus (A); hard palate (H); soft palate (S). If the letter is capitalized it means that the cleft is complete; if lower-case it means it is an incomplete cleft. If the letter is on the right it means that the cleft is on the right side and vice versa (Shah et al., 2011).

2.3.4 International Classification of Diseases (ICD)

The International Classification of Diseases (ICD) is a World Health Organization's (WHO) classification used worldwide as the standard diagnostic descriptive tool for epidemiology, health management and clinical purposes. One of the major classifications is congenital malformations of eye, ear, face and neck that includes OFC (Hill, 2014). In 1995, the WHO published a book entitled 'Application of the ICD to dentistry and stomatology' (ICD-DA).

It concerns all diseases and conditions that occur, manifest or are associated with the oral cavity and adjacent structures. It provides a convenient classification for all those working in the field of dentistry. OFC is classified into three categories starting with: Q35 for CP; Q36 for CL; and Q37 for CLP. Appendix A2 lists the codes for NSOFC sub-phenotypes according to ICD classification version 10 (World Health Organization, 2015).

2.3.5 Other rare NSOFC sub-phenotypes

This category includes a group of NSOFC phenotypes that are rarely diagnosed. They have not been investigated by this study but, for the sake of completion, are described here.

2.3.5.1 Submucous CP:

Submucous CP is a mild type of CP wherein there is insufficient medial fusion of the muscles of the soft palate hidden under the mucous or midline notch at the posterior edge of the bony palate. It occurs is 1/2500–5000 births (Gosain et al., 1996). There are only limited studies that deal with this sub-phenotype of CP due to difficulties in diagnosis and the potential for misdiagnosis in many situations. One case-control study was conducted on 103 German patients with submucous CP to investigate possible environmental and genetic risk factors. Among the 12 candidate genes that were investigated, *TGFB3* and *MNI* showed significant association with submucous CP. Furthermore, a significant association with maternal smoking was identified in this study (Reiter et al 2012).

2.3.5.2 Microform cleft lip (MCL):

MCL is a rare type of OFC that has the characteristic appearance of corrected CL. It is considered a CL that has healed intrauterinally. MCL is usually accompanied by a notch in the vermilion border and a 'collapsed' nostril. MCL could result from either a partial failure in the fusion of the fronto-nasal and maxillary processes before week seven of embryonic

life, or from a spontaneous late foetal repair of an open cleft lip (Francesconi et al., 2003; Castilla and Martinez-Frias, 1995).

2.4 Normal development of OFC

The development of orofacial segments is a complex processes that involve multiple steps.

2.4.1 Formation of the lip and primary palate

The development of the lip and palate occur between the 4th and the 15th week of embryogenesis, following precise genetic regulatory cascades that direct the migration and proliferation of the neural crest mesenchyme cells to form the pharyngeal arches. Arising from the first pharyngeal arch dorsal portions where the fronto-nasal prominence is shaped, the maxillary processes are formed in the 4^{th} week and then extend forward beneath the eye. In the 5^{th} week, the nasal prominences are shaped from thickening of the ridges surrounding the nasal pits on either side. From the outer edge of the nasal pits, the lateral nasal prominences are formed; and from the inner edge of the nasal pits, the medial nasal prominences are formed. The two medial nasal prominences grow and bridge over the maxillary prominence laterally and fuse together (Figure 2.2). They also merge medially and deeply forming together a prominence known as the intermaxillary segment. This consists of a labial component, which forms the philtrum of the upper lip; an alveolar component that carries the four incisors; and a jaw component that forms the primary palate. From the maxillary processes, two shelves grow out in the 6th week of development and are directed obliquely downward on either side of the tongue. Anteriorly, the palatal shelves fuse with the primary palate to complete orofacial development (Sperber, 2002).

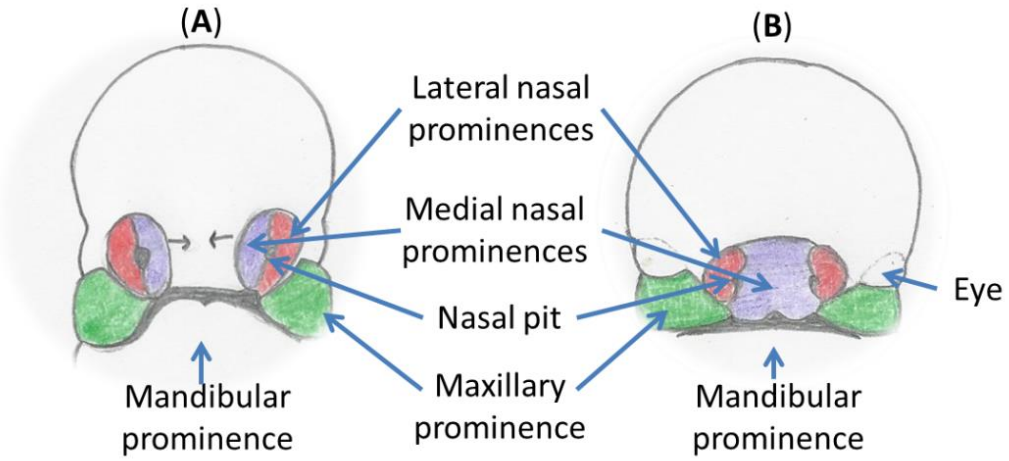


Figure 2.2: Frontal view of the face. (A) Five-week embryo. The nasal prominence tissue ridge surrounds the nasal pits and forms the medial and lateral nasal prominences. (B) Seven-week embryo. The medial nasal prominences from each side fuse with the maxillary prominence. They also merge medially and deeply to fuse together forming the intermaxillary segment.

2.4.2 Formation of the secondary palate

The secondary palate consists of the hard and soft palate. It is called the secondary palate because it is formed after the primary palate, which forms the philtrum and anterior portion of the alveolar bone. Formation of the soft and hard palates start in the 7th week of development from the maxillary processes which consist of two shelves on either side of the tongue. In the 8th week, the palatine shelves change their direction horizontally above the tongue (see Figure 2.3). This elevation is critical and occurs in a short period of only few hours during the 8th week. It is controlled by many factors, including: upright movement of the foetal head, away from the heart prominence, which allows the jaw to open and the tongue to move downward; and hypoglossal muscle along with jaw movement, directed by the pharyngeal arch muscles, which together depress the tongue downward (Wragg et al., 1972; Diewert, 1985). Foetal flow in amniotic fluid allows the jaw to grow freely.

Furthermore, the forward growth of the mandible provides space for the tongue to move. If mandible growth is retarded, as occurs in Pierre Robin sequence, clefting may result (Lavrin and Hay, 2000). Elevation of the palatine shelves is also affected by gender; male shelf elevation occurs a few days earlier than female elevation.

Finally, the two palatine shelves fuse with each other from the anterior of the hard palate to the posterior of the soft palate, forming the secondary palate. These processes are completed by the 10th week (Sperber, 2002).

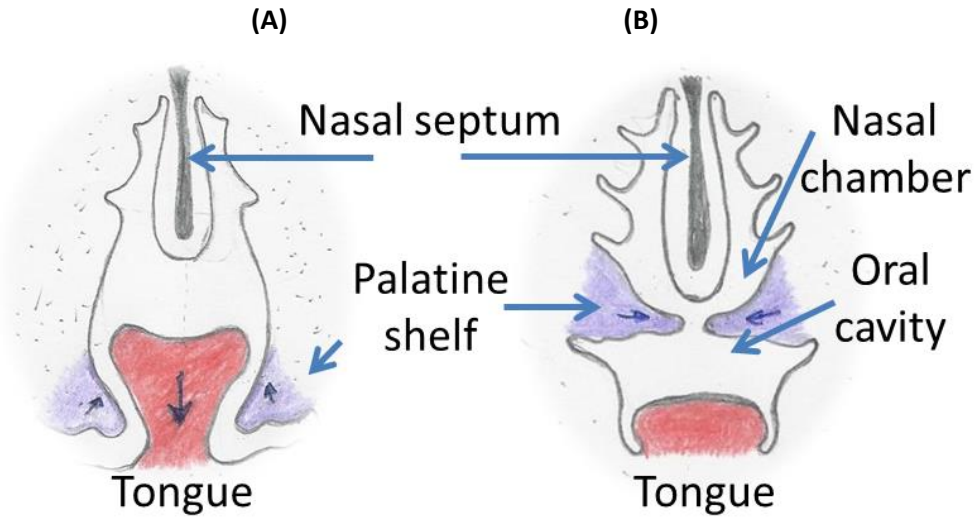


Figure 2.3: Frontal section through the head. (A) Seven-week embryo with a palatine shelf located on either side of the tongue. (B) Eight-week embryo. The palatine shelves change direction above the tongue.

2.5 Molecular biologic control of lip and palate development

The developmental steps of the lip and palate are initiated, controlled, and regulated by many genes, growth factors, growth receptors, and local changes that interact together. If any of these components or interactions are disturbed through environmental factors, direct

interference, gene mutation or deficiency of one of the controlling elements, cleft lip and/or palate might result (Thomason et al., 2008; Ashique et al., 2002; Sun et al., 2000).

2.5.1 Primary palatogenesis

Molecular studies have shown that the initiation, identification and development of the orofacial region is controlled by fibroblast growth factors (FGFs), sonic hedgehog (SHH), bone morphogenetic proteins (BMPs), the homeobox containing genes *BRX1* and *MSX1*, the distal-less homeobox-containing (*DLX*) genes, and local retinoic acid gradients. Only a few limited studies have investigated the molecular control of primary palate fusion, most of which were carried out on mice. However, it has been suggested that molecular controlling factors included *SHH, MSX1, MSX2, BMP, FGF*, and *TP63* (Sun et al., 2000; Ashique et al., 2002; Thomason et al., 2008). Other studies have identified different transcription factors, growth factors and their receptors including Osr2, Lhx8, Msx1, BMP4, SHH, BMP2, Fgfr2b, Tgfb2, Tgfbr2 and Fgf10 (Gritli-Linde, 2007b).

2.5.2 Elevation of the palatal shelves

The molecular control of palatal shelf initiation and vertical growth includes a complex of signalling incorporating transcription factors, growth factors and their receptors, many of which were listed above e.g. Osr2, Lhx8, Msx1, Fgf10, Fgfr2b, Tgfb2, and Tgfbr2 (Gritli-Linde, 2007a). Moreover, interacting signals between the palatal epithelium and the mesenchyme have been suggested to regulate palatal growth as well (Mossey et al., 2009).

Several explanations have been suggested to account for the initiation of palatal shelf elevation above the tongue. A sudden increase in the tissue fluid might initiate the elevation by enhancing rapid growth; muscular movement; or an intrinsic shelf force resulting from

biochemical transformations in the physical consistency of the connective tissue matrix of the shelves, including vascularity and blood flow variation. In addition, the synthesis, hydration and accumulation of hyaluronic acid glycosaminoglycan within the extracellular matrix has also been suggested to play a role in shelf elevation. Moreover, collagen fibres and mesenchymal cells are oriented and organised in the palatal shelves in a way that suggests they may affect and direct the elevating force (Brinkley and Morris-Wiman, 1987; Meng et al., 2009).

2.5.3 Fusion of the palatal shelves

The fusion of the two palatal shelves with the primary palate is a crucial step for the completion of palatal development. The epithelium covering the palatal shelves thickens. Apoptotic cell death, phagocytosis, and removal of the epithelial cells in the targeted area are initiated but this process is not clearly understood. Gaps in the basal lamina, separation of epithelial cells and evidence of mesenchymal cell migration have been found in the fusion site. Some epithelial cells undergo mesenchymal transformation to display a fibroblastoid phenotype and thereafter stay at the fusion site or, in a later step, migrate laterally to participate in forming connective tissue (Martínez-Álvarez et al., 2000a; Martínez-Álvarez et al., 2000b). Glycoproteins and desmosomes cover the degenerating epithelial cells and facilitate palatal fusion. However, desmosomes and hemidesmosomes have been found to be minimal in this region, suggesting that their role is not relevant for the adhesion of the palatal shelves (Abbott, 2010). The cell adhesion molecule syndecan is expressed while the shelves are being elevated; expression subsequently decreases during fusion. (Fitchett, 1990). After fusion, the epithelial and mesenchymal cells on the oral cavity side differentiate into oral

mucosa (stratified squamous epithelium). Molecular studies have suggested a few factors that control this process including MSX1, MSX2, SHH, TGFB3, BMPs and FGFs.

In the following paragraph we will discuss the different approaches used by molecular studies to identify genes involved in the aetiology of oral clefts.

2.6 Genetic aetiological studies for NSOFC

Genetic aetiological studies have used several approaches to assess genes responsible for or involved in the development of oral clefts. These include candidate gene approach and genome-wide association studies (GWAS). The following section will discuss genes that were analysed using these two approaches.

2.6.1 Candidate gene approach

Candidate gene analysis relies upon choosing a known gene that is related to pathways thought to be implicated in the aetiology of the disorder under study, especially those that regulate gene expression or development and that have detectable levels of expression in relevant embryonic tissues in human and/or mouse models (Dixon, 2011; Juriloff and Harris, 2006; Brown et al., 2003). OFC syndromes and rare Mendelian cleft syndromes are considered valid targets for the candidate gene approach in NSOFC for the potential causal reasons discussed above, and because candidate gene analysis has an established history of success with these categories of disorders (Zucchero et al., 2004; Jugessur et al., 2009; Dixon, 2011). Another method for selecting candidate genes is through cytogenetics; wherein genes are prioritized because of their location in abnormal (e.g. deleted; rearranged)

chromosomal regions identified in patients with the disorder (Brewer et al., 1999; Gong et al., 2005; Higgins et al., 2008).

Several genes have been linked to NSOFC. These genes have been classified according to their functional classes that include: transcription factors; extracellular signalling factors; growth factors; and cell signalling (Mossey, 2001a; Mossey et al., 2009).

2.6.1.1 Transcription factors:

Transcription factors are genes that code for proteins, which bind to specific DNA sequences, regulating the expression (transcription) of their target genes (Latchman, 1997). Genes in this class play a role in the aetiology of NSOFC include: Msh homeobox 1 (*MSX1*); Interferon regulatory factor 6 (*IRF6*); Ventral anterior homeobox 1 (*VAX1*); Small ubiquitin-like modifier (*SUMO*); Forkhead box E1 (*FOXE1*); V-maf musculoaponeurotic fibrosarcoma oncogene homolog B (*MAFB*); Tumour protein 63 (*TP63*); and ATP-binding cassette transporter 4 (*ABCA4*).

2.6.1.1.1 Msh homeobox 1 (MSX1) 4p16:

MSX1 is one of the genes responsible for initiation and growth of the facial processes and specification of their identity. It also plays an important role in craniofacial development as it is required for the activation of *BMP2* and *BMP4* in the palate mesenchyme (Zhang et al., 2002). Signals from the mesenchyme during the growth of the palatal shelves regulate the expression of *BMP2* and *BMP4* in the mesenchyme, *SHH* and *BMP4* in the medial edge epithelium (Van den Boogaard et al., 2000; Gritli-Linde, 2007b).

Van den Boogaard et al. (2000) found that mutations in *MSX1* in humans are associated with OFC and congenital missing teeth. Furthermore, Jezewski et al. (2003) found from sequencing the *MSX1* gene that 2% of the NSOFC patients in his study had *MSX1* mutations

(Hu et al., 2003; Jezewski et al., 2003). Furthermore, Jagomägi et al. (2010) genotyped 18 candidate genes in a case control study in Estonia and found a statistically significant relationship between NSOFC and *MSX1* for 5 Single nucleotide polymorphism (SNP) (rs11726039, rs868257, rs6446693, rs1907998, and rs6832405).

2.6.1.1.2. Interferon regulatory factor 6 (IRF6) 1q32.2:

IRF6 was the first identified NSOFC susceptibility locus (Mangold et al., 2010). It has been the only candidate gene consistently shown to have a significant association with NSOFC across multiple studies (Marazita et al., 2004; Jugessur et al., 2008; Jagomägi et al., 2010). Studies have shown that *IRF6* is expressed from the epithelium in the ectodermal edge of the palatal shelves during and just before primary and secondary palate formation (Ben et al., 2005; Washbourne and Cox, 2006; Knight et al., 2006; Jugessur, 2009). *IRF6* is also expressed during palatal shelf fusion (Kondo et al., 2002). *IRF6* has thus been proposed to play an important role in controlling epithelial cell proliferation and differentiation (Ferretti et al., 2011). Consistent with this hypothesis, *IRF6* null mice have abnormal skin, limb, and craniofacial development (Kondo et al., 2002).

IRF6 mutation is responsible for two autosomal dominant OFC syndromes, Van Der Woude and Popliteal pterygium (Kondo et al., 2002; Jugessur et al., 2009; Lima et al., 2009). Van Der Woude syndrome is an OFC syndrome that resembles NSOFC; however, in most cases it includes an additional clinical feature, a lip pit. Additionally, although syndromic, it is caused by mutation of the *IRF6* gene alone.

Rahimov et al. (2008) have identified a SNP (rs642961) that, if mutated, causes OFC through disruption of a transcription factor AP-2α binding site in an *IRF6* enhancer element. This SNP has also been reported to be an important element for cranial closure and orofacial

development (Schorle et al., 1996). Furthermore, mutation of *TFAP2A*, which is the gene encoding AP-2α, causes a syndrome that has features similar to Van Der Woude syndrome (Milunsky et al., 2008).

Multiple *IRF6* SNPs have been suggested to be associated with NSOFC. In Estonia, the *IRF6* rs590223 SNP was found to be significantly associated with NSOFC (P= 0.039) (Jagomägi et al., 2010). In China, Li et al. (2012) assessed the association between *IRF6* rs2235371 and CL/P in a case-control study of 106 patients. They carried out Transmission Disequilibrium Test (TDT), Family Based Association Test (FBAT), and Haplotype-Based Haplotype Relative Risk (HHRR) analyses but found no significant differences in rs2235371 genotype frequencies (GG, GA and AA) between cases and controls (P> 0.05). However, there was a statistically significant difference in allelic frequencies (P< 0.05). There was also a statistically significant difference in both genotype and allele frequencies of rs2235371 variants between family members of CL patients and controls. TDT analysis suggested a linkage in the presence of disequilibrium (P= 0.024). Results of HHRR analysis (P= 0.024) and FBAT (P= 0.027) also indicated an association between *IRF6* rs2235371 variants and the risk of NSOFC (Li et al., 2012).

In west China, Huang et al. (2009) assessed associations between three *IRF6* SNPs (rs2013162, rs2235375, and rs2235371) and NSOFC, using both case-parent trio and case-control designs with 332 NSOFC cases. They found a statistically significant over-transmission of the rs2235371 common 'C' allele (P= 0.013) and under-transmission of the rs2235375 'C' allele (P< 0.001). There were statistically significant differences between cases and controls in the frequencies of the rare rs2235371and rs2235375 alleles. *IRF6* rs2013162 was not related to NSOFC in their population. Furthermore, Blanton (2010a), who examined *IRF6* variant/NSOFC associations in different ethnic groups, found a

significant association between the *IRF6* rs2235371 common allele and NSOFC in Non-Hispanic Whites compared to Hispanics. In addition, Birnbaum et al. (2009) found a decrease chance of having an infant with NSOFCwith rs2235371 common allele (C) (OR: 0.367 and 95% CI: 0.163 to 0.823) in a population of Central European origin. In a hospital-based case-control study carried out in Chinese Han, Pan et al. (2010) found that the rs2235371 rare homozygous allele (TT) and heterozygous (CT) genotypes were associated with decreased risk of NSOFC compared to the common allele (CC). They also reported higher risk of NSOFC when two polymorphisms (rs642961 rare allele (A) and rs2235371 common allele (C)) were combined. Furthermore, Zucchero et al. (2004) found a strong relationship between *IRF6* (rs2235375 and rs2013162) and NSOFC in Italian, American (Texan), Belgian, and Asian (Taiwan and Singapore) populations using TDT analysis.

A recent study in Northeast China assessed the relationship between NSOFC and 12 SNPs in 7 candidate genes using an allele-specific primer extension technique for case-parent (236 patients, 185 mothers and 154 fathers); 128 complete trios; and case-control (400 controls) analyses. The *TGFA* and *IRF6* genes showed a statistically significant association with NSOFC. *IRF6* had a statistically significant association between rs2235371 (P= 0.003), rs2013162 (P= 0.001) and NSOFC. Furthermore, LD analysis found tight linkage between the SNPs in *IRF6* and *TGFA*. Haplotypes of the four *TGFA* SNPs (rs3771494, rs1058213, rs11466285, and rs3771523) and two *IRF6* SNPs (rs2013162 and rs2235371) were also determined. In addition, FBAT showed a significant over-transmission of the rs2235371 common allele (C) in CL/P trios (P= 0.007) (Lu et al., 2013).

2.6.1.1.3 Ventral anterior homeobox 1 (VAX1) 10q25.3:

The *VAX1* gene encodes a transcriptional regulator with a DNA-binding homeobox domain. It is a member of the *Emx/Not* gene family and is mainly expressed in optical regions.

However, Vax1 is also expressed widely in developing craniofacial structures (Bertuzzi et al., 1999). Recently, VAX1 was found to be located near a locus of high GWAS significance (10q25) in two studies using GWAS case-control parental trios (Beaty et al., 2010; Mangold et al., 2010). Beaty et al. (2010) carried out a GWAS a CL/P case-triad study on 825 trios of European ancestry and 1,038 trios of Asian ancestry. They reported that among the 13 SNPs in *VAX1* analyzed, two approached genome-wide significance (rs7078160 and rs4752028) in both TDT and conditional logistic regression tests (Beaty et al., 2010). *VAX1* rs7078160 also reached a genome wide significant relationship in CL/P cases ($P= 1.92 \times 10^{-8}$) in a GWAS study using a central European population (Mangold et al., 2010). Relevance of *VAX1* to NSOFC is also supported by the observation that the *VAX1* null mouse showed CP (Hallonet et al., 1999). Therefore, it is expected that variants of the *VAX1* gene might play a role in the aetiology and pathogenesis of cleft lip and palate.

In a case-control candidate gene approach study, Rojas-Martinez et al. (2010) investigated whether *IRF6* (rs861020), and SNPs located in 8q24 (rs987525), 10q25 (rs7078160) and 17q22 (rs227731), regions associated with NSOFC, are implicated in the aetiology of NSOFC (CL/P) in non-Europeans (Mayan Mesoamerican population) This study confirmed an association between *IRF6*, 8q24, and 10q25 and NSOFC (CL/P) in the Mesoamerican population.

In a case-control study by Slavotinek et al. (2012), *VAX1* was sequenced in 70 patients, leading to the first demonstration of homozygosity of two nucleotides in a patient, who was of Egyptian origin and consanguineous parents. His clinical feature included microphthalmia, small optic nerves, cleft lip/palate, and corpus callosum agenesis, thus providing further evidence for a relationship of *VAX1* to NSOFC. Another case-control

study, by Nasser et al. (2012), studied 384 patients with non-syndromic CL/P. They sequenced 17 rare *VAX1* variants, not including rs7078160 and rs4752028. They suggested that although there were no significant differences found in their study between cases and controls, *VAX1* was more likely to be associated with CL/P (Nasser et al., 2012).

Ludwig et al. (2012) identified six new risk loci from their meta-analysis carried out on two GWAS studies on non-syndromic CL/P. One was the rs7078160 SNP on 10q25, which showed a TDT P-value that reached genome-wide significance ($P= 3.96*10^{-11}$ for European and Asian trios and $P= 2.81*10^{-8}$ for European trios).

In 2013, Butali et al. replicated two of the GWAS signals using 1,326 individuals in European and Asian populations. TDT analysis found a strong association between CL/P and *VAX1* rs7078160 ($P= 2.7*10^{-6}$) and *VAX1* rs475202 (P= 0.0002) for the combined Asian sample. They also found a statistically significant effect of a parental rare allele, with preferential maternal ($P= 6.5 \times 10^{-5}$) versus paternal transmission (P= 0.004) (Butali et al., 2013).

2.6.1.1.4 Small ubiquitin-like modifier (SUMO) 2q33.1:

SUMO is a post-translational modification protein that modifies cellular proteins and plays a role in some cellular processes like transcriptional regulation, apoptosis and protein stability. Alkuraya et al. (2006) found that the *SUMO* gene was disrupted in a balanced reciprocal translocation between chromosomes 2q33 and 8q24 in a female patient born with unilateral CLP. Furthermore, they found strong *SUMO1* expression in the upper lip and the palate and in an established embryonic stem cell line. The study suggested a specific role for *SUMO1* in the network of genes regulating palatal development. Shi et al. (2009) identified *SUMO1* gene microdeletions following examination of 333 candidate genes in 2823 NSOFC patients. Similar results were reported by Carter et al. (2010). In addition, *SUMO* has been reported to

be a gene that is susceptible to environmental changes such as stress. A relationship also exists between *SUMO* and several genes that have been reported to have a strong association with OFC such as several in the FGF signalling pathway, *MSX1*, *TP63*, *TBX22*, *SATB2*, *TRPS1* and *EYA1*. Therefore, early modification of *SUMO* during pregnancy as a result of environmental effects might lead to NSOFC indirectly through its impact on other genes directly responsible for NSOFC (Andreou et al., 2007; Pauws and Stanier, 2007; Jugessur et al., 2009).

2.6.1.1.5 Forkhead box E1 (FOXE1) 9q22:

FOXE1 is part of the forkhead/ winged-helix family, which are transcription factors and key regulators in embryogenesis. Deficiency of the *FOXE1* gene in mouse models leads to a severe CP (De Felice et al., 1998). In human, mutation and loss of function of *FOXE1* is responsible for Bamforth-Lazarus Syndrome, which includes CP as one of its clinical features (Clifton-Bligh et al., 1998; Castanet et al., 2002; Brancaccio et al. 2004). In 2005, Vieira et al. sequenced 184 NSOFC patients in a matched case-control study and found mutations in *FOXE1* in two patients compared to none in the controls. Furthermore, a GWAS study in 2009 found a significant linkage signal in 9q21 that is close to the *FOXE1* locus (Marazita, 2009).

2.6.1.1.6 V-maf musculoaponeurotic fibrosarcoma oncogene homolog B (MAFB) 20q12:

MAFB is a leucine zipper transcription factor that regulates gene expression. Recently *MAFB* was suggested to be associated with NSOFC by the GWAS GENEVA Cleft Study as its locus was near markers that achieved genome-wide significance (Beaty et al., 2010). This relationship could be related to the fact that MAFB is a leucine zipper that is responsible for binding to other genes associated with OFC such as *IRF6* and *MSX1*. However, this relationship showed higher significance among trios of Asian rather than European origins.

In addition, a missense mutation in a highly conserved region was found in 3.5% of Filipinos with NSOFC compared to 0.7% in the controls, suggesting the presence of a rare variant of *MAFB* in Asians (Beaty et al., 2010; Dixon 2011).

2.6.1.1.7 Tumour protein 63 (TP63) 19q13.43:

TP63 is a gene expressed in the branchial arch and it plays a role in induction of factors involved in epithelial-mesenchymal signalling in craniofacial development. Mutation of *TP63* is responsible for five syndromes, four of which carry OFCs as a clinical feature. OFC resulting from *TP63* mutation has been reported to be associated with other congenital defects and syndromes (Rinne et al., 2007). However, Leoyklang et al. (2006) have found mutations in *TP63* in isolated cleft cases as well. The distribution of mutations along the gene was reported by Rinne et al. (2007) to have a clear genotype-phenotype relationship.

Recently, researchers have studied the relationship between *IRF6* and *TP63* expecting that both genes work together during palatal fusion. They found that *IRF6* is a direct transcriptional target of TP63 and that both proteins work in a regulatory feedback loop necessary for proper epithelial proliferation and differentiation. (Moretti et al., 2010; Thomason et al., 2010; Romano, 2012). In their study on the conserved Pbx-Wnt-p63-Irf6 regulatory module in mice, Ferretti et al. (2011) reported a direct regulatory effect of TP63 on *IRF6* in orofacial development. They also suggested a Pbx-Wnt-p63-Irf6 module, which induces apoptosis at the embryonic junction between the nasal and maxillary processes. Interruption of this process could cause NSOFC. This model is also supported by Kurosaka et al. (2014), who reported an association between interruption of SHH signalling and the aetiology and pathogenesis of cleft lip through antagonistic interactions with other gene regulatory networks, including TP63/IRFf6 signalling pathways.

2.6.1.1.8 ATP-binding cassette transporter 4 (ABCA4) 1p22.1:

ABCA4 is a gene responsible for the synthesis of a protein that is found in the retina and, if mutated, causes an autosomal-recessive retinal degenerative disease. Although *ABCA4* achieved genome-wide significance in the GENEVA cleft GWAS study (Beaty et al., 2010), with stronger evidence among trios of Asian origin, *ABCA4* is not detected in the palatal shelves in mouse models. Therefore, it was suggested that the signal detected in the GWAS study was likely related to another gene close to *ABCA4* locus (Bille et al., 2007; Beaty et al., 2010).

2.6.1.2 Extracellular signalling factors and growth factors:

Extracellular signalling factors are responsible for the regulation of the secretion of the extracellular matrix, which provides structural support and adhesive substrates for body tissues. The extracellular matrix plays a significant role in regulating the behaviour of cells, cell shape and movement, and facilitates cell-cell and cell-matrix interactions (Schnaper and Kleinman, 1993).

2.6.1.2.1 Fibroblast Growth Factors (FGF):

FGF signalling pathway family members play an important role in craniofacial development including neural crest induction, skeletogenesis, and epithelial mesenchymal induction (Nie et al., 2006). Many studies have linked members of the FGF family and it's receptors to OFC.

2.6.1.2.1.1 Fibroblast growth factor receptor 1 (FGFR1) 8p11:

FGFR1 was found to be related to CP (Trokovic et al., 2003). FGFR1 is a growth factor that is associated with the autosomal dominant Kallmann syndrome, which presents with OFC as

an occasional clinical feature. However, in some cases OFC might be the only or the first diagnostic feature (Dode et al., 2003; Dodé & Hardelin, 2009).

2.6.1.2.1.2 Fibroblast growth factor 8 (FGF8)10q24:

FGF8 is expressed during neural crest migration. When mutated, *FGF8* was found to be related to CP in conjunction with multiple other defects in studies carried out in mice (Abu-Issa et al., 2002).

2.6.1.2.1.3 Fibroblast growth factor 10 (FGF10) 5p13:

FGFR2b receptors receive signals from the palate mesenchyme, located in the palate epithelial region, to regulate the growth of the palatal shelves and maintain Sonic Hedgehog signalling-regulated expression in the palate. Failure in any of these steps leads to clefting of the palate (Rice et al., 2004; Mossey et al., 2009).

2.6.1.2.2. Transforming growth factor (TGF):

Transforming growth factor (TGF) family members play an important role in craniofacial development; proliferation, differentiation and apoptosis of epithelium and mesenchyme

2.6.1.2.2.1 TGF-β1,2 and 3 (14q24):

TGF-βs are a family of structurally related growth and differentiation factors that influence all important cellular process from proliferation, differentiation and apoptosis remodelling through epithelial-mesenchymal transformation (Abbott 2010). They are involved in regulating skeletal development including cartilage and bone formation and patterning of mesoderm and craniofacial development (Wan and Cao, 2005). *TGF-β*s are expressed at early stages in the developing palate and *TGF-β3* is expressed in the epithelium of the vertically growing palatal shelves (Fitzpatrick et al., 1990). *TGF-β3* continues to be expressed in the epithelium after elevation until adhesion and fusion starts as it plays an important role in this process (Abbott et al., 2005). The epithelium of the two elevated and

contacting palatal shelves and of the basement membrane remains intact in *TGF-β3* deficient mice, resulting in CP in these animals (Proetzel et al., 1995; Kaartinen et al., 1997). Furthermore, knocking-out *TGF-β3* inhibited palatal shelf fusion in mice (Nawshad et al., 2004). Murillo et al. (2009) confirmed that *TGF-β3* has an active role of in medial edge epithelium cell death and suggested a role of *TGF-β1* in apoptotic clearance of medial edge epithelium cells to allow for palatal fusion.

Tang et al. 2013 carried out a meta-analysis that included eleven case-control studies to investigate the relationship between TGF-β3 genetic polymorphisms and NSOFC risk in human. They reported the possibility of TGF-β3 gene polymorphisms contributing to NSOFC, especially among Asian populations

2.6.1.2.2.*2 TGF-α* (2p13):

TGF-α is expressed in palatal tissue especially in the midline and subjacent mesenchyme of the palatal shelves during palatal fusion (Dixon et al., 1991). In his review (covering 1986 to 2005) on the evidence for involvement of TGFα in the aetiology of OFC, Vieira (2006) has suggested that *TGFα* acts as only a small risk factor for NSOFC. A 20% increase in the prevalence of NSOFC in infants carrying a rare *TGF-α* variant was also reported, leading to the suggestion that *TGF-α* may act as a modifier rather than being a determinant gene for NSOFC (Vieira, 2006).

In 2014, Lu et al. carried out a meta-analysis of 29 studies to investigate the association between *TGF-α* and NSOFC. A significant increase in the risk of NSOFC was reported with a *TGF-α*/*Taq*I polymorphism (OR: 1.70 and 95% CI: 1.41 to 2.05) and a reduced risk with a *GFA*/*Bam*HI polymorphism rare allele (OR: 0.44 and 95% CI: 0.30 to 0.64). This study also suggested that *TGF-α* plays a role in the aetiology of NSOFC.

2.6.1.2.2.3 Bone morphogenetic protein (BMP):

BMP is another member of the TGF-β family. The *BMP* gene encodes the bone morphogenic protein (BMP) that binds to membrane bound receptors (serine/threonine kinases) entering into a series of receptor translocations and activation of specific target genes. They play a role in mediating the interaction between epithelium and mesenchyme during the development stage of the embryo (Hogan 1996). Furthermore, *BMP* (14q22.2) interacts with *MSX1* in palatal development as the MSX1 protein causes activation of *BMP4*. A study in embryonic mice has shown that *BMP4* mutation led in particular to bilateral CL in all embryos on the 12th day of conception. However, most of the clefts fused in the subsequent 14 day as other bone morphogenic factors (BMFs) are available to complement and cross regulate the deficiency caused by the mutated gene (Liu et al., 2005; Jiang et al., 2006). Therefore, this gene could be less important than other members of the group as its effect could be overcome.

2.6.1.2.3 Hyaluronan Synthase isozymes 1, 2 and 3 (HAS1, 2 and 3) 19q13.3-q13.4, 8q24.12 and 16q22.1:

Three *HAS* genes (*HAS 1, 2* and *3*) are responsible for the synthesis of hyaluronan in the palatal shelves just before their elevation. Hyaluronan becomes the major component of the shelves, resulting in a swelling of extracellular matrix and contributing to the elevation of the palatal shelves in mouse models studies (Toole, 1997). In humans, *HAS* deficiency causes embryonic death or stillbirth (Girish and Kemparaju, 2007), and therefore the *HAS* genes have not been considered as candidates for OFC in population and case-control studies.

2.6.1.3 Cell signalling:

Gene activation and regulation of cell signalling functionality are responsible for controlling information transmission into and within a cell. Information is transported either through

protein-protein interactions or it is transmitted by diffusible elements usually referred to as second messengers (Berridge, 2012). This category of cell signalling includes the folate pathway (methylenetetrahydrofolate reductase (MTHFR) pathway) which influences nutrient metabolism.

2.6.1.3.1 Folate pathway (Methylenetetrahydrofolate reductase (MTHFR) 1p36.3:

MTHFR produces methylene-tetrahydrofolate reductase, which is an enzyme that reduces the level of homocysteine. MTHFR converts 5,10-methylenetetrahydrofolate to 5-methylenetetrahydrofolate, which is the methyl donor for remethylation of homocysteine into methionine (Wong et al., 1999). In addition, MTHFR play an important role in folic acid metabolism. It is responsible for catalysing tetrahydrofolate to methylene- tetrahydrofolate by the addition of methylene groups. Tetrahydrofolate is produced by the reduction of folic acid to dihydrofolic acid by the enzyme dihydrofolate reductase; dihydrofolic acid is then further reduced to tetrahydrofolate (Frosst et al., 1995).

Pan et al. (2012) carried out a systematic review and meta-analysis to assess the relationship between the *MTHFR* C677T variant and NSOFC among Asian and Caucasians populations. The search yielded 17 case-control studies. Among Asian populations, *MTHFR* C677T homozygous rare allele and heterozygous genotypes in infants were more highly associated with NSOFC versus common allele homozygotes (CC): (OR: 1.741 and 95% CI: 1.043 to 2.907) for CT versus CC; OR: 2.311 and 95% CI: 1.313 to 4.041 for TT versus CC; and OR: 1.740 and 95% CI: 1.051 to 2.882 for CT/TT versus CC, respectively). Similar results were observed for the *MTHFR* C677T ‘T’ allele, when using the ‘C’ allele as a reference in Asians (OR: 1.420 and 95% CI: 1.191 to 1.693, for ‘T’ versus ‘C’ alleles). Furthermore, in analyses stratified by disease types, CT/CC was suggested to reduce susceptibility to CL/P under a recessive genetic model (OR: 0.854, 95% CI: 0.730 to 1.000). For Caucasians, the *MTHFR*

1298C allele in the case group was of significantly lower frequency than that in the control group, suggesting a reduced chance of having an infant with NSOFC for this allele (OR: 0.711 and 95% CI: 0.641 to 0.790, for 'C' versus 'A' alleles).

Recently, a systematic review and meta-analysis of Asian populations that further considered the association between *MTHFR* (C677T and A1298C genotypes) and CL/P by updating the previous meta-analysis and adding papers that was included. Nine case-control studies met the inclusion. The met-analysis showed a significant relationship and increase in the risk of CL/P in children (OR: 1.41 and 95% CI: 1.23 to 1.61) and mothers (OR: 1.70 and 95% CI: 1.19 to 2.42) with *MTHFR* C677T genotypes (TT versus CC) (Zhao et al., 2014). Furthermore, a case-control triads study in China found a significant relationship between *MTHFR* and NSOFC, but only in Northern China, suggesting an etiological variant (Zhu et al., 2010). Also, Schultz et al. (2004) study which was not included in the meta-analysis, scanned 50 genomic regions for association with NSOFC, and found a significant relationship between over-transmission of the *MTHFR* C677T allele in 36 families from the Philippines (P= 0.01).

Therefore, MTHFR 677T rare allele seems to be associated with an increased risk of CL/P with an ethnic variation.

The candidate gene approach have preliminary listed multiple genes associated with NSOFC. Confirmation of their association can be achieved either through having several studies concluding a positive interaction or by GWAS study.

2.6.2 Genome-wide association studies (GWAS)

Genome Wide Association (GWA) is an approach that involves rapid scanning for markers across the complete sets of DNA, or genomes, of many people and is used to find genetic variations associated with a particular disease. Several GWAS parental triad case-control studies were carried out on different populations to identify possible genes involved in birth defects. Birnbaum et al. (2009) conducted a GWAS triad case-control study involving 224 CL/P and 383 controls of Central European origin. 8q24 showed highly significant markers that reached genome wide significance with a P= 3.34×10^{-24}, OR: 2.57 and 95% CI: 2.02 to 3.34. Grant et al. (2009) performed a case-control cohort study in Philadelphia using 111 study subjects and 5951 controls of European descent and confirmed a strong association between the 8q24 locus (rs987525) and CL/P. However, to date this locus does not harbour a characterized gene. Furthermore, Beaty et al. (2010) carried out 'the GENEVA study' on Asian and European ethnic groups, including 1908 case-parent trios (825 European, 1038 Asian, and 45 African), and identified genome wide significant SNPs on 8q24 (rs987525), *IRF6* (rs2073485, rs2013162, rs8610020, rs10865790), *MAFB* 20q12 (rs6072081, rs6065259, rs17820943, rs13041247, rs11696257, and rs102085), *ABC4* (1p22.1) (rs4147811, rs481931, and rs560426) and SNPs approaching genome wide significance on 10q25 (*VAX1*) (rs7078160 and rs4752028), *PAX7*, and *NTNI*. Another GWAS in the same year was carried out in Germany using 401 CL/P, 1,323 controls and 793 triads, and identified two loci that reached genome wide significance (10q25.3 and 17q22). Two genes have been reported to be near the 10q25.3 locus: *VAX1* and KIAAI598 (Mangold et al., 2010).

Therefore, loci identified in NSOFC GWAS studies include;

2.6.2.1 8q24.21:

8q24 is a susceptibility locus that has been previously reported to be strongly associated with OFC in genome-wide association studies (Mangold et al., 2010). Although no gene has been identified in this region (Grant et al., 2009; Blanton et al., 2010b), the strength of the association indicates the region to be highly important.

Birnbaum et al. (2009) conducted a study to look for a susceptibility locus for CL/P in 146 SNPs on 8q24 in a GWAS case-control and case-control family-based study. In the GWAS study, three SNPs were identified as having the most significant markers (rs987525, rs17241253 and rs1530200). In the case-control family-based study, only one SNP reached a significant level (rs987525). This SNP also reached a highly significant result in a Mostowska et al. (2010) study of the Polish population. In addition, in his GWAS study on CL/P patients of European decent, Grant et al. (2009) reported a strong genome-wide significant relationship between SNP rs987525 and CL/P, with P= 9.18×10^{-8}, OR: 2.09 and 95 % CI: 1.59 to 2.76.

In his family based study on Hispanic and non-Hispanic white families, Blanton et al. (2010b) studied six SNPs located in 8q24 and found a strong association with non-Hispanic whites but not with Hispanics. Furthermore, the 8q24 association reached significance in a Mesoamerican population in Mexico. Rojas-Martinez et al. (2010) similarly carried out a case-control study to investigate whether the *IRF6*, 8q24, 10q25, or 17q22 loci contribute to CL/P in a Mesoamerican population. Their finding confirmed the relationship between both 8q24 and 10q25 with CL/P.

Pan et al. (2011) aimed to replicate three novel susceptibility loci findings including rs987525 on 8q24 that was reported to be significantly related to NSOFC in previous

research on European Caucasians (Birnbaum et al., 2009; Mangold et al., 2009). The study included 199 NSOFC patients compared to 210 controls in a Chinese Han population. However, the frequency of the rs987525 SNP variant in this population was not sufficient to be effectively genotyped (Pan et al., 2011). This finding was supported by Murray et al. (2012), who investigated the association between 8q24 and CL/P in both European and Asian populations. They reported a low power of association between 8q24 and CL/P in the Asian population that was suggested to be due to lower variant allele frequencies in Asians (Murray et al., 2012).

2.6.2.2 10q25:

The 10q24 locus has reached a genome-wide significance level in several NSOFC/ GWAS studies (Beaty et al., 2010; Mangold et al., 2010). A significant association of 10q25 with NSOFC was also confirmed in Scandinavian (Norway and Denmark), Mesoamerican, Mayan origin and Polish populations in a case-control study (Rojas-Martinez et al., 2010), and in a Baltic population case-control study (P= 0.0016) (Nikopensius et al., 2010).

In their case-control study that assessed a replication of two of the novel loci (10q25 and 17q22), Pan et al. (2011) found no significant difference between cases and controls for 10q25 in China's population. A more recent case-control study (2012) on 206 Polish patients analysed chromosomal regions located at 1p22.1, 10q25.3, 17q22, and 20q12. Significant results were observed in 10q25.3 (rs7078160 and rs4752028) and in 17q22 (rs227731). Both rs7078160 and rs4752028 were associated with more than a four-fold increase in the risk of CLP (P= 0.001, OR: 4.53 and 95% CI: 1.678 to 12.265; and OR: 4.57 and P= 0.0004, 95% CI: 1.81 to 11.51; respectively) (Mostowska et al., 2012).

These were the main GWAS studies and their identified loci associated with NSOFC. Further GWAS studies are recommended in different populations and for different NSOFC phenotypes to verify their genetic risk factors.

This was a brief review of the literature on two of their main approaches used to identify genes responsible for NSOFC. However, in order to carry out genotyping for these genes, several field and laboratory steps are required. The following section discusses these steps.

2.7 DNA, collection, extraction, analyzing and genotyping

The methodology of genetic aetiological studies involves; DNA extraction, DNA analysis, polymerase chain reaction (PCR) and/or sequencing and genotyping. This section discusses these steps

2.7.1 Sample collection

DNA can be extracted from EDTA-anticoagulated whole blood, buccal swabs, cheek swabs, or saliva.

2.7.1.1 Whole blood samples:

Whole blood sampling has the following advantages and disadvantages;

Advantages:

1. Greater DNA quality and quantity sufficient for genome-wide research that requires approximately 20 micrograms of high quality DNA.
2. Offers the potential for saving plasma or serum for analysis of other analytics, such as micronutrients or for storing cells for subsequent RNA or protein studies.

Disadvantages:

1. Invasive procedure with challenges in acquisition from subjects especially small infants (blood draw from healthy 'control' infants is generally discouraged).
2. Available quantities might not be sufficient especially from infants.

2.7.1.2 Buccal or cheek swabing:

Buccal swabs and cheek swabbing have the following advantage and disadvantages;

Advantages:

1. Easy to collect and acquire.

Disadvantages:

1. Quality and quantity might not be sufficient.
2. Potential contamination by food debris/micro-organisms.

2.7.1.3 Saliva samples:

Saliva sampling has the following advantages and disadvantages;

Advantages:

1. Relatively easy to acquire and collect.
2. Usually provides a sufficient amount of DNA, depending on (a) method used to obtain sample and (b) quantity of saliva required.

Disadvantages:

1. Age dependent (more difficult in infants).

2. Expensive.

2.7.2 DNA Extraction

After saliva is collected, DNA is extracted and isolated, with the goal of purifying the DNA sample (Genomic DNA purification). The process involves several steps:

- Disruption and lysis of the cells to open and expose the DNA using chemical and physical means;
- Removal of proteins and contaminants by digestion with proteinase K; and

- Recovery of DNA using ethanol.

Purified DNA is ready to undergo amplification through the polymerase chain-reaction (PCR) or freezing and storage for future analysis. Purified DNA can remain stable for several years.

2.7.3 Polymerase chain-reaction (PCR)

PCR is a technique used to replicate a specific region of DNA. Its main advantage lies in the rapid amplification of a small region of interest in the DNA in a, generally, sequence-independent manner. Starting with single stranded DNA, PCR replaces the missing strand using Taq DNA polymerase, starting from an annealed primer in a manner similar to that by which DNA normally undergoes replication (Figure 2.4).

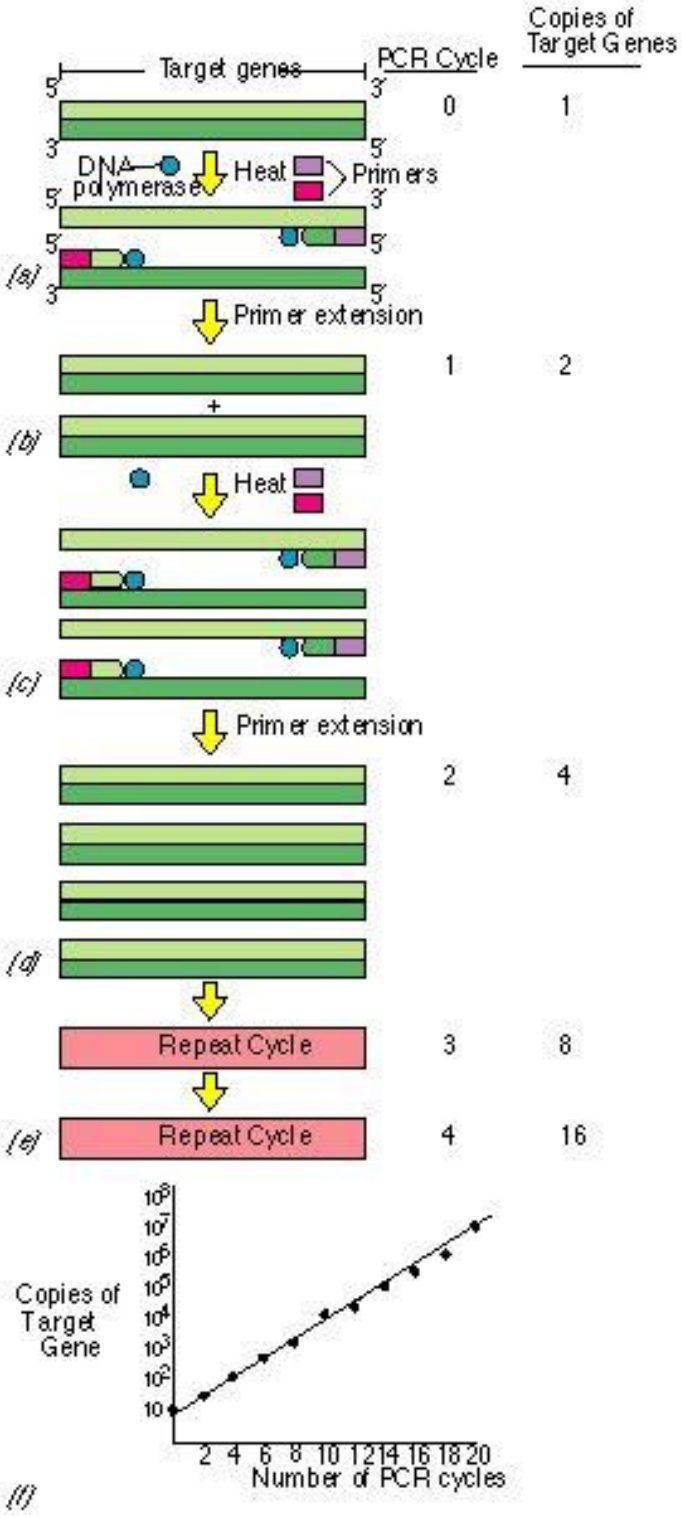


Figure 2.4: Diagram for the PCR method (adapted from Madigan et al., 1999). The target DNA is amplified through three steps: first (a), the DNA is heated to produce single strands (denaturing); second (b), primers are added that are complimentary to the ends of the target DNA; third (c,d), the primer extension is catalysed by the Taq DNA polymerase enzyme to produce a new strand. The 3 steps are repeated (e) until the desired amount of product is reached (f).

2.7.4 PCR with restriction-digestion enzymes

The restriction digestion procedure is used subsequent to PCR amplification of DNA, and involves using restriction endonuclease enzymes to cleave the DNA at sites with a specific nucleotide sequence. The restriction enzymes are chosen to recognize a sequence motif containing either the normal or variant allele, which is then cleaved (or not) depending on the experimental design. For example, if the enzyme recognizes the DNA strand carrying the variant site, the DNA carrying the common allele will not be cut. (Pritchard and Korf, 2008). The DNA fragments carrying a sequence difference can then be differentiated by size or other means. This technique has the advantage of being simple and inexpensive to perform in studies where known mutations are being investigated.

2.7.5 Real-time PCR

Real-time PCR is used for detecting and/or relative quantitating variant sequence using fluorescent probes (oligonucleotides with attached fluorescence resonance energy transfer molecules) that are complimentary to each strand of (usually) the variant sequence in the gene after amplification of the exons. The process is applied during or following polymerase replication, depending on specific methodologies used. This procedure has the advantage of being less expensive than direct genome sequencing and is easier to apply than restriction-enzyme digestion after PCR.

2.7.6 Sequencing

Genomic DNA sequencing provides a complete transcript of the DNA strand or fragment in order to detect any site of error or mutation, traditionally using fluorescent labelled nucleotides and a DNA polymerase reaction mix. The end-result will appear as a ladder of bands that is read directly from a single lane capillary gel (Figure 2.5).

Genome-sequencing is indicated when the location or type of gene mutation is unknown, scanning of large gene segments is needed or there are scattered mutations along the gene. However, it is not recommended when the goal is to investigate a limited repertoire of known mutations, as time, expense are major concerns.

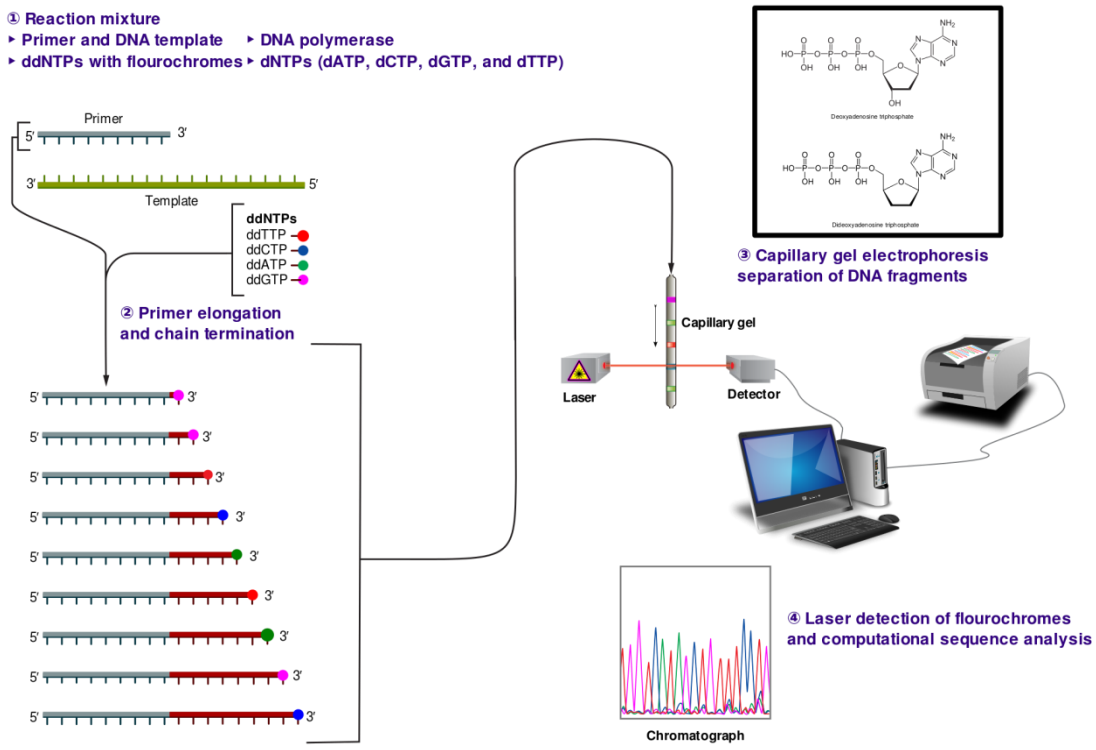


Figure 2.5: Diagram of DNA sequencing method. The DNA to be sequenced is mixed with a fluorescent labelled primer (ddNTPs) complementary to the 3' end of the sequence. Four parallel specific reaction mixes, which contain the four normal nucleotide precursors with a single fluorescent terminating nucleotide in each mix. The DNA fragments produced are then separated by capillary electrophoresis and the sequence read with a laser beam. (Adapted from: http://commons.wikimedia.org/wiki/File:Sanger-sequencing.svg)

2.8 Environmental factors related to OFC

Environmental risk factors were reported to play an important role in the aetiology of oral clefts. Previous research has proposed a number of factors, including; socioeconomic status, maternal medication ingestion, infections, contact with chemicals/ smoking during the first trimester and consanguinity. In the following section the environmental risk factors related to NSOFC will be discussed in the light of previous literature.

2.8.1 Demographic variables

This section discusses demographic variables that have been linked to NSOFC

2.8.1.1. Gestation age, weight and twin pregnancies:

Czeizel (2002) reported no significant relationship between NSOFC and gestation age. However, a significant association between lower birth weight and NSOFC has been reported (Czeizel, 2002; Jia et al., 2011). This apparent discrepancy has been explained as arising from a retardation of intrauterine growth resulting from an alteration in the activity of TGF (Czeizel, 2002). Twin pregnancies have been reported to be associated with birth defects (Glinianaia et al., 2008). Furthermore, Nordström et al. (1996) reported a higher prevalence of monozygotic (versus dizygotic) twins with NSOFC. They also reported a higher prevalence of twin pregnancies in CP.

2.8.1.2 Parental age:

Increased parental age has been suggested to be associated with an increase in birth defects. Elahi et al. (2004) found a statistically significant relationship between NSOFC and parental ages ($P< 0.05$). Furthermore, Rajab and Thomas (2001) reported that the mean parental age

of infants with NSOFC in Oman were significantly greater than that of the general population (P< 0.001). Bille et al. (2005) reported paternal age to be a risk factor for NSOFC but not maternal age. Furthermore, Zandi and Heidari (2011) reported that maternal age was not a risk factor for development of NSCLP (Zandi and Heidari, 2011). On the other hand, Jia et al. (2011) found a significant difference in maternal age between cases and controls P< 0.001. In 2012, Herkrath et al. carried out a meta-analysis on the relationship between OFC and parental age using the data from 13 studies and found no significant relationship. However, they suggested that the risk of NSOFC is increased to 58% when fathers are older than 40 years and 20 to 29% higher when mothers are older than 35 years (Herkrath et al., 2012). Further investigation is needed to verify this suggestion and include studies that were not included in the meta-analysis.

2.8.2 Socioeconomic status (SES)

Familial SES could be measured by several ways. It could be estimated by looking at certain dimensions such as; family income, parental occupation, parental education and the description of the family neighbourhood (Bornstein, 2014). These points are discussed in the following section.

2.8.2.1: Parental education

Furthermore, Krapels et al. (2006) and Carmichael et al. (2009) reported significantly lower maternal and paternal education in NSOFC cases compared to controls. According to Krapels et al. (2006) paternal education is associated with CL/P (OR: 1.6 and 95% CI, 1.0 to 2.3) and CP (OR: 4.5 and 95% CI: 2.1 to 9.4). However, maternal education was associated with CL/P (OR: 1.6 and CI: 1.1 to 2.3) (Krapels, 2006). Further studies are needed to clarify

the association between paternal education and NSOFC in different geographic locations, and whether there are any other factors associated with this relationship as part of SES of the infant.

2.8.2.2 Parental occupation

Occupation could be related to NSOFC in several ways: (a) through affecting the SES of the family or (b) through occupational exposure to hazardous substances. Studies have reported occupations that may result in maternal exposure to certain chemicals such as aliphatic aldehydes, ethyl-ether, aliphatic acids, trichloroethylene and pesticides. Maternal exposure to these chemicals could result in offspring with congenital abnormalities (Desrosiers et al., 2012; Garlantézec et al., 2009; Chevrier et al., 2006; Lorente et al., 2000). A population-based case-control study in Norway found an association between parental occupation and NSOFC (Nguyen et al., 2007). For maternal occupation, there was a statistically significant relationship with mothers working as hairdressers (for CL/P: OR: 4.8 and 95% CI: 0.99 to 23) and in manufacturing and in food production (for CL/P: OR: 3.8 and 95% CI: 1.3 to 11, and for CP: OR: 7.1 and 95% CI: 1.5 to 33). For paternal occupations, no significant relationship was reported between woodworking and CL/P (OR: 1.7 and 95% CI: 0.85 to 3.2) or CP (OR: 2.0 and 95% CI: 0.82 to 4.7); whereas paternal housekeepers showed an increased CP only (OR: 12 and 95% CI: 3.3 to 46) (Nguyen et al., 2007). Mirilas et al. (2011) reported a significant relationship between CL/P and farmers (P= 0.039, OR: 3.00 and 95% CI: 1.03 to 8.70). On the other hand, Czeizel (2002) found no significant relationship between parents working with acrylonitrile and having an offspring with NSOFC. It therefore seems from the limited research available that maternal occupation is more related to CP than to CL/P. However, further investigation is needed to verify the relationship between parental occupation and NSOFC phenotypes.

2.8.2.3 Description of the family neighbourhood

Alsahafi (2010) had described SES as a risk factor for NSOFC in Saudi Arabia and found that although there was lower SES for families with NSOFC children, this relationship was not statistically significant. However, he reported a statistically significant higher risk of OFC at birth in children living in rural areas compared to controls (P< 0.001 and 95% CI: 4.2 to 42.9).

In addition, Messer et al. (2010) assessed the variation of oral clefts (CL/P and CP) across Texas urban-rural areas from 1999 to 2003. They reported an increased CL/P risk in rural areas compared to urban areas. On the other hand, Stoll et al. (1991) found no significant differences in the prevalence of both CL/P and CP, in rural compared to urban areas. Further investigation in different geographic region is needed to verify the relationship.

2.8.3 Pregnancy planning and the effect of sibling order in the family

Pregnancy planning, parental health care, and affected sibling order in the family are all related to maternal healthcare, which could affect infants. In a Netherlands case-control study, Krapels (2006) reported a 50% decrease in NSOFC risk in planned pregnancies (OR, 0.5; 95% CI, 0.2 to 0.99). In addition, Mossey et al. (2007) reported a significant decreased risk of NSOFC after pregnancy planning in the United Kingdom (Scotland and the Manchester and Merseyside regions of England) from 1997 to 2000 in a case-control study utilizing 191 case/247 control participants (OR: 0.51 and 95% CI: 0.33 to 0.79). In Saudi Arabia, Alsahafi (2010) reported a 1.3-fold increased chance of NSOFC in offspring of unplanned pregnancies (OR:1.3, CI: 0.7 to 2.3), a doubled risk in children who were 4th or

later in birth order (P= 0.02, OR: 2.0, CI: 1.1 to 3.6), and born to families with less parental-physician interaction than the controls (P= 0.6).

Studies have shown that birth order may be associated with birth defects including NSOFC. Rajab and Thomas (2001) conducted a case-control study in Oman, where they found a significantly increased chance of NSOFC in younger versus older siblings. Vieira and Orioli (2002) carried out a meta-analysis on published data discussing the relationship between birth order and NSOFC from 1966 to 2000. They found that children with higher birth order are more likely to have NSOFC. In addition, they reported an increase in NSOFC risk with increased birth order with an OR peak of 3.0 in children with a birth order of 4th or higher. The conclusion of this meta-analysis suggested the need for further studies taking into consideration sample size and factors that might influence birth order such as income status, paternal age, and vitamin intake (Vieira and Orioli, 2002). However, a more recent study (Martelli et al., 2010) used a case-control design to evaluate environmental risk factors for CL/P in 100 children in Minas Gerais. They reported no relationship between CL/P and paternal age, pregnancy order or interpregnancy interval.

2.8.4 Dietary factors and supplementation

Nutritional factors and supplementations could have a direct effect on preventing NSOFC, or an indirect effect through interactions with other nutrients or genes or through epigenetic effects. Because of theses possible mechanisms, many dilatory factors and supplements have been investigated for their role in NSOFC.

2.8.4.1 Dietary factors

Nutritional factors have been suggested to play an important role in oral cleft prevention. In their case-control study in the Netherlands, Krapels (2005) concluded that a higher

preconception intake of nutrients with predominant amount of fruits and vegetables reduced the risk of having a child with NSOFC. She also reported that intake of vegetable protein, fibre, ascorbic acid, iron, and magnesium might provide a reduced chance of having an infant with NSOFC. Neural tube defect (NTD) and NSOFC are similar in origin as the developmental origins of the structures that are malformed in both conditions arise from neural crest cells. Therefore, it has been suggested that vitamins such as folic acid and multiple vitamins, which prevent NTD might also prevent NSOFC. Other nutrients that could be related to NSOFC are Vitamin A, zinc, ascorbic acid, β-carotene, α-tocopherol, pantothenic acid, biotin, iron, and magnesium (Mossey, 2001a). Conversely, it has been suggested that a maternal diet with a high fat content might induce NSOFC in mice (Zhou and Walker, 1993). A systematic review carried out to investigate the effect of multivitamins supplements and folic acid fortification on birth defect reported on papers from January 1966 to July 2005. They reported a significant decrease chance of having an infant with CL/P in case-control studies (OR: 0.63 and 95% CI: 0.54 to 0.73) and CP (OR: 0.76 and 95% CI: 0.62 to 0.93); but was not significant in cohort and randomized controlled studies (Goh et al., 2006).

Recently, Wallenstein et al. (2013) carried out a population based, case-control study in California from 1999–2003 that included 170 CP, 425 CL/P and 534 control individuals. They investigated the association between the intake of vitamin supplements and dietary nutrients and the risk of developing CL/P and CP during maternal periconception. Generally, a reduced risk of NSOFC was observed with maternal ingestion of vitamin supplements. In addition, they reported a twofold elevated risk of CP with low intake of riboflavin, magnesium, calcium, vitamin B12, and zinc and a twofold elevated risk of CL/P with low intake of niacin, riboflavin, vitamin B12, and calcium.

The best way to measure the association between nutrient intake and birth defects is by analysing the levels of nutrient biomarkers. The advantage of certain biomarkers is that their blood levels after pregnancy could be a useful indicator of the levels of the nutrient during the first week of pregnancy. Moreover, maternal biochemical markers could be useful even years following delivery if used in case-control studies (Munger et al., 2011). Therefore, studies that aim to measure the association between nutrient intake and NSOFC by nutrient biomarkers are recommended.

2.8.4.2 Folic acid:

Folic acid has been suggested to prevent NSOFC through its role in remethylation of homocysteine into methionine and S-adenosylmethionine by carbon donation (Krapels, 2005). Lin et al. (1989) who examined the effect of folate deficiency on homocysteine metabolism through giving rats folate-deficient diet, reported an increase in levels of homocysteine, up to four-fold in rats folate-deficient diet compared to the control group with normal diet. If homocysteine does not undergo recycling, it alters embryo development by affecting neural crest cell migration leading to malformations (Rosenquist et al., 1996). In a human case-control study, Wong et al. (1999) found a higher level of homocysteine in NSOFC mothers as compared with controls. Another case-control study carried out in the Netherlands measured red blood cell, serum folate and plasma homocysteine concentration in venous blood sample of infants. They found 15% lower level of mean serum folate levels in infants with NSOFC compared to controls but the differences were not significant (P= 0.06) (van Rooij et al., 2003a).

In 2002, a Hungarian intervention cohort based case-control study which was not included in Molina-Solana et al. (2013) meta-analysis, reported no relationship between folic acid and

NSOFC when using an 0.8 mg dose combined with multivitamins (Czeizel, 2002). However, in his Hungarian Case-Control Surveillance of Congenial Abnormality (HCCSCA) study he found a decrease chance of having an infant with NSOFC when using folic acid in high dose (6 mg per day), which could indicate a dose-dependent protective effect (Czeizel et al., 1999). However, Wilcox et al. (2007) conducted a national population based case-control study in Norway and found that folic acid supplementation (≥400 μg/day) reduced the risk of CL/P but not CP after adjustment of multivitamins and smoking (OR:0.61 and 95% CI: 0.39 to 0.96). Also, Krapels (2005) case-control study in the Netherlands found about 40% reduction in the chance of having an infant with NSOFC when ingesting a minimal dose of 400 μg per day of folic acid supplementation

Molina-Solana et al. (2013) carried out a systematic review and meta-analysis to 2011, investigating the effect of environmental factors, including maternal ingestion of folic acid supplementation during pregnancy, on the incidence of CL/P. Twenty-eight case-control studies were analysed, seven of which discussed folic acid. An overall decrease chance of having an infant with CL/P was reported with an OR of 0.78 and 95% CI: 0.62 to 0.97. Following, a case-control study in China, Jia et al. (2011) reported a decrease chance of having an infant with NSOFC of maternal folic acid supplementation but this was statistically significant only for CP (P= 0.004, OR: 0.52, 95% CI: 0.33 to 0.81).

Although the preliminary results suggest a relationship between folic acid and NSOFC, description of the effective dose, duration and timing of the supplementation ingestion, still need further investigation. In addition, research needs further to clarify and confirm to which NSOFC phenotype folic acid is related.

2.8.4.3 B Vitamins:

The B vitamin group contain important factors for various metabolic processes involved in the synthesis of normal DNA and RNA, critical for normal body development. Vitamin B1 (thiamine) is involved in the metabolism of amino acids and carbohydrates (Thurnham, 2000; Russell, 2001). Vitamin B2 (riboflavin) is involved in fat, carbohydrate and protein metabolism as well as in folate metabolism (Rivlin, 1970). Vitamin B3 (niacin) is involved in the synthesis of pentose, steroids, red blood cells, and fatty acids and in the metabolism of proteins, carbohydrates and fats as well as glycolysis. In addition, vitamin B3 alters the metabolism of certain drugs and toxins (Thurnham, 2000; Russell, 2001;). Vitamin B6 (pyridoxine) is involved in the metabolism of carbohydrates, fats, amino acids and glycogen. It also acts as a cofactor for the trans-sulphuration of homocysteine to cysteine. In addition, vitamin B6 is involved in regulating the activity of hormones that bind to the nuclear receptor and influence gene expression (Jacobsson and Granstrom, 1997; Thurnham, 2000; Russell, 2001). Therefore, a suggestion of alteration of the levels of B vitamins during pregnancy has been suggested to be associated with NSOFC.

Krapels et al. (2004b) reported that dietary intake of energy and of vitamin B was lower in mothers of NSOFC compared with controls. This relationship was significant for vitamin B1, vitamin B3, vitamin B6 and energy (P= 0.007, P= 0.01, P= 0.007 and P= 0.04, respectively). They also reported that there was a decrease in NSOFC risk of about 21-64% when consumption of vitamin B1 was above 1.08 mg per day. For vitamin B6, the decrease in the NSOFC risk was 29-65% when consumption was above 1.51 mg per day.

Li et al. (2009) conducted a case-control study to measure the effects of vitamin B6 supplementation against having an infant with NSOFC. They injected pregnant mice with toxic material that caused OFC and administered one of the groups with vitamin B6.

Although OFC rates were low in animals administered vitamin B6, the difference was not significant. Munger et al. (2004) also reported a decrease chance of having an infant with NSOFC when ingesting vitamin B6 supplementation in humans. van Rooij et al. (2003a) concluded that low serum levels of vitamin B6 and vitamin B12 in mothers increased the risk of NSOFC in infants. Vitamin B1was also reported to reduce the risk of NSOFC (Krapels et al., 2004b; Munger et al., 2004).

2.8.4.4 Vitamin A:

Vitamin A has a specific inhibitory effect on cranial crest cells, from which the development of the craniofacial structure originates (Teratology Society, 1987). The relationship between OFC and Vitamin A is inconsistent. It has been suggested that vitamin A supplementation might reduce the risk of NSOFC, although it is teratogenic in high doses (Czeizel and Rockenbauer, 1998; Soprano and Soprano, 1995). In Norway, enhanced vitamin A levels through supplementation and diet has been shown to reduce the risk of CP by about 53% (Johansen et al., 2008).

2.8.4.5 Zinc (Zn):

Low levels of zinc are associated with multiple malformations, as zinc has a role in the absorption of folate and is involved in conversion of 5-methylene tetrahydrofolate into tetrahydrofolate (Krapels, 2005). Zinc is also a cofactor for several metalloenzymes, is a constituent of proteins, hormones and neuropeptides, and is important for cellular multiplication, differentiation and apoptosis, which are integral in embryonic development. It also has a role in genetic control of embryonic development (Tamura and Goldenberg, 1996). Hurley and Swenerton (1966) have reported that 34% of foetal rats had CP after their mothers received diets containing low levels of zinc during gestation. In human, the relationship between zinc and NSOFC was analysed through measuring the serum

concentration of zinc among NSOFC mothers or infants and comparing them with controls (Krapels et al., 2004b; Tamura et al., 2005).

Molina-Solana et al. (2013) carried out a meta-analysis on four case-control studies assessing the relationship between zinc and CL/P. In general, the meta-analysis reported no significant relationship between zinc serum concentration and CL/P (OR 1.82; 95% CI 0.88 to 3.79). However, there was a tendency of relationship that needs further investigation in future studies with larger sample size.

2.8.4.6 Calcium:

Calcium has also been suggested to have an effect on NSOFC development. In a case-control study from China, a significant decrease chance of having an infant with CL/P was reported for calcium supplementation , (P= 0.019, OR: 0.66 and 95% CI: 0.47 to 0.93) (Jia et al., 2011). Recently, Wallenstein et al. (2013) reported a decrease in the risk of CP and CL/P associated with maternal ingestion of calcium supplementation in the pre-gestation period in his population based case-control study, but the relationship was not significant (OR: 0.5, and 95% CI: 0.2 to 1.3 for CP and OR: 0.7, and 95% CI: 0.4 to 1.4 for CL/P).

2.8.4.7 Food fortification:

Fortification of flour with folic acid has been suggested as a source of nutrient and a means to prevent birth defects. Examination of the effects of flour fortification in Chile showed a significant decrease in the prevalence of neural tube defects. However, there was no significant reduction in NSOFC, which might indicate that folic acid only has a limited effect in the prevention of NSOFC (Cortés et al., 2012).

In 2008, Johnson and Little carried out a systematic review and meta-analysis to assess the role of folate, including folate fortification, in the aetiology of NSOFC. Seven studies were

analysed in which five measured compulsory fortification and two measured optional fortification. Compulsory fortification was found to have a mild effect (OR: 0.93 and 95% CI: 0.90 to 0.98 for CL/P and OR: 0.92 and 95% CI: 0.85 to 0.99 for CP) whereas no effect was identified following optional fortification (OR: 1.02 and 95% CI: 0.93 to 1.12) in relation to the prevalence of NSOFC (Johnson and Little, 2008).

In Saudi Arabia, the site of the current study, the National Flour Mills Organization ordered mandatory folate fortification of flour starting in 2001 (Arabic date: 1421) with the minimum requirement being 1.653% (16.53 grams of folic acid for per kilogram of flour) (Saudi Standard of fortification, 1421H (2001)). The other sources of flour in Saudi Arabia are the flours that are imported from Kuwait and the UAE which are also fortified.

2.8.5 Maternal medication and illness

Studies have investigated the association between maternal exposures to medication and illness, with both CL/P and CP. In the following paragraphs we will discuss the possibility of this association and the different medications involved:

2.8.5.1 Maternal medication:

Maternal exposure to medication has been suggested to play a role in the development of NSOFC. Zandi and Heidari (2011) have confirmed a significant relationship between maternal drug ingestion, trauma and exposure to radiation with CLP.

2.8.5.1.1Antibiotics

Antibacterial agents including antibiotics have also been reported to be associated with NSOFC (OR: 2.1 and 95% CI: 1.2 to 3.9) (Crider et al., 2009). In a cohort study, Molgaard-Nielsen and Hviid (2012) investigated the association between maternal antibiotic ingestion and the risk of having an offspring with NSOFC, but found no association. However, when

further classification of antibiotic usage was performed, they found a significantly increased risk for infant CL/P for mothers taking doxycycline/tetracycline (OR: 7.30 and 95% CI: 1.81 to 29.46) and sulfamethizole (OR: 1.76 and 95% CI: 1.10 to 2.81), specifically. Furthermore, there was an increased risk of CP in offspring of mothers taking trimethoprim (OR: 14.29 and 95% CI: 3.46 to 59.05) or using pivmecillinam (OR: 2.34 and 95% CI: 1.20 to 4.54). In addition, Lin et al. (2012) carried out a case-control study on 877 NSOFC cases to investigate the relationship between maternal amoxicillin ingestion in early pregnancy and NSOFC occurrence. They found an increased risk for CL/P (OR: 2.0 and 95% CI: 1.0 to 4.1) and CP risk (OR: 1.0 and 95% CI: 0.4 to 2.3) in offspring. For ingestion during the third-gestational-month the risk was higher for both CL/P (OR: 4.3 and 95% CI: 1.4 to 13.0) and for CP (OR: 7.1 and 95% CI: 1.4 to 36). Further investigation to clarify the relationship between antibiotics and NSOFC is required.

2.8.5.1.2 Corticosteroid

Carmichael et al. (2007a), conducted a case-control study on 1141 NSOFC cases to measure the relationship between maternal corticosteroid ingestion during pregnancy and the chance of having an infant with NSOFC. They concluded that there was a moderately increased risk of infants with CL/P in mothers using corticosteroids (OR: 1.7 and 95% CI: 1.1 to 2.6) but not CP (OR: 0.5 and 95% CI: 0.2 to 1.3) (Carmichael et al., 2007a). Other studies support the relationship between maternal corticosteroid ingestion and NSOFC (Pradat et al., 2003; Rodriguez-Pinilla and Martinez-Frias, 1998). On the other hand, Chi et al. (2013) conducted a retrospective cohort study on 2658 pregnant women exposed to topical corticosteroids compared to 7246 controls, and found no significant relationship between NSOFC and maternal topical corticosteroid ingestion (Chi et al., 2013). This finding is also is supported by other studies (Kallen, 2003; Czeizel and Rockenbauer, 1997). Recently, Skuladottir et al, (2014) investigated maternal first-trimester exposure to corticosteroids and oral clefts in

offspring using two population-based studies. Thy reported inconsistent finding for the two populations suggesting that meta-analyses will be required to determine whether a relationship between NSOFC and maternal corticosteroid ingestion exists.

2.8.5.1.3 Antithyroid drugs

The relationship between antithyroid drugs and oral cleft were also studied. Andersen et al. (2013) found a significant relationship between maternal antithyroid drug ingestion and malformations in the face and neck region of their offspring in a nationwide register-based cohort study on 817,093 live-births from 1996 to 2008 in Denmark ($P < .001$). However, future cohort or longitudinal studies to identify any teratogenic effects of antithyroid medication ingested by pregnant mothers is needed to confirm this supposition.

EUROCAT (European Surveillance of Congenital Anomalies), a multicentre European network of registries for the epidemiologic surveillance of congenital anomalies, has established policies (Scope of Policy Actions Needed for Primary Prevention of Congenital Anomalies) to be considered for the primary prevention of congenital anomalies when developing guidelines for national plans and strategies on rare diseases, including guidelines on medication ingestion. They recommend appropriate medical and clinical care for pregnant women starting from the preconception period especially for those at a high-risk of epilepsy, diabetes, as well as those who require medication (EUROCAT, 2013; Little, 2004).

2.8.5.2 Maternal illness:

Maternal illness has been suggested to have an effect on embryonic development (Mossey, 2001a). Maternal illness is accompanied by infection, inflammation, high fever and the ingestion of medication. The association between maternal illness and birth defects could be explained by direct effects from infection of the foetus or indirect effects from hyperthermia, toxic metabolites, or the side effects of medications (Edwards, 2006). A case-control study in

Hungary reported a higher occurrence of CL/P in mothers affected by herpes, whereas their medication did not show an association with clefting (Czeizel, 2002). A maternal common cold infection in the first trimester was reported to significantly increase the risk of CLP in girls (OR: 3.3 and 95% CL: 1.6 to 7.1), but no effect was found in boys (Krapels, 2005). In addition, maternal fever has also been reported to increase the risk of CL/P (OR: 1.28 and 95% CI: 1.01 to 1.63), and mothers who took antipyretic drugs showed a lower risk of NSOFC than did mothers who did not control their fever (Hashmi et al., 2010).

Diabetes in pregnancy has been reported to increase the risk of having infants with neural defects and NSOFC (Spilson et al., 2001), which could be explained by the possible effects of hyperglycaemia on gene expression (Fine et al., 1999). Finally, Murphy et al. (2013) carried out a meta-analysis on cohort studies from 1975 to 2012 to determine the association between maternal asthma and birth defects. After analysing 21 studies that met their inclusion criteria, they found a significantly increased risk of CL/P in offspring of mothers with asthma (RR: 1.30 and 95% CI: 1.01 to 1.68). This association could be related to maternal steroid consumption during critical period of CL/P and CP development.

The effect of maternal diseases on orofacial structure development still needs further investigation to clarify the relationship and its pathophysiology.

2.8.6 Maternal life-style

Maternal life style factors are suggested to influence the development of oral clefts such as; weight changes, smoking, and alcohol consumption

2.8.6.1 Maternal weight changes:

Maternal weight changes could be related to the aetiology of NSOFC. Jia et al. (2011) reported that a decline in maternal weight during pregnancy was associated with a higher risk of having an infant with NSOFC (P< 0.005) and increased maternal weight had a lower risk of having an infant with CL/P (OR: 0.20 and 95% CI: 0.06 to 0.60) and CP (OR: 0.2 and CI: 0.045 to 0.56). On the other hand, maternal obesity has been related to birth defects such as spina bifida and anencephaly (Waller et al., 2007). A systematic review and meta-analysis was carried out by Stothard et al. (2009) to assess the relationship between maternal overweight status and congenital anomalies including NSOFC. Thirty-nine articles were reviewed and eighteen were analysed in the meta-analysis, which identified a significantly increased risk of CP (OR: 1.23 and 95% CI: 1.03 to1.47) and CL/P (OR: 1.20 and 95% CI: 1.03 to 1.40) in obese mothers. In addition, Block et al. (2013) conducted a study of the relationship between maternal pre-pregnancy body mass index (BMI) and 26 birth defects identified through the Florida Birth Defects Registry. They found a direct dose-response relationship between maternal pre-pregnancy BMI and both CL/P and CP (Block et al., 2013). Further studies to clarify the relationship between maternal weight and weight changes and NSOFC are needed.

2.8.6.2 Smoking:

Previous studies have consistently identified a positive association between maternal smoking and NSOFC (Hackshaw et al., 2011; Zandi and Heidari, 2011). A 2004 meta-analysis has suggested a modest positive association between active smoking and NSOFC: OR: 1.34 and 95% CI: 1.25 to 1.44 for CLP; and OR: 1.22 and 95% CI: 1.10 to 1.35 for CP (Little et al., 2004b).

In 2011, another systematic review and meta-analysis was conducted to clarify the relationship between smoking and the prevalence of birth defects and included studies from the previous meta-analysis. Literature was reviewed from 1959 to 2010 and 172 articles were used in the meta-analysis. Following odds ratio (OR) adjustment for maternal age and alcohol consumption, a significant relationship between smoking and NSOFC was reported (OR: 1.28 and 95% CI: 1.20 to 1.36) (Hackshaw et al. 2011). In addition, Meyer et al. (2004) reported a dose-response effect in smoking/ CP association. Such an effect was also suggested by Little et al. (2004a) in their case-control study designed to investigate the relationship between smoking and orofacial clefts in the United Kingdom. They found that maternal smoking of more than 10 cigarettes per day increased the chance of having an infant with NSOFC from (1.5 to 2) to (2.5 to 3) fold for the various NSOFC phenotypes (Little et al., 2004a). Moreover, Honein et al. (2007) found a statistically significant relationship between maternal smoking and NSOFC (OR: 1.3 and 95% CI: 1 to 1.6). However, when relating maternal smoking to cleft severity, a stronger association was reported with bilateral CL/P (OR: 1.7 and 95% CI: 1.2 to 2.6).

On the other hand, although there has been a decrease in maternal smoking in Denmark in the last 20 years, the prevalence of NSOFC has not decreased (Bille et al., 2005). Also, in a French case-control study conducted in 2008 it was reported that although a significant relationship between maternal smoking and NSOFC was not identified, there was a statistically significant relationship and an increased chance of NSOFC offspring with maternal exposure to environmental tobacco smoke (Chevrier et al., 2008). Therefore, a systematic review and meta-analysis including fourteen case-control studies, on the relationship between passive smoking and NSOFC has been carried out by the author and colleagues. It concluded that maternal passive smoking exposure approximately doubles the

risk of having a child with NSOFC (OR: 2.11, 95% CI: 1.54 to2.89) (Sabbagh et al., published/ in press 2014). The paper is presented in (appendix B2)

Paternal smoking has also been reported to increase the risk of NSOFC by 1.5 fold (Krapels, 2005), and specifically of CL/P (OR: 1.92 and 95% CI: 1.40 to 2.64) and CP (OR: 2.09, 95% CI: 1.40 to 3.13) (Jia et al., 2011).

From these studies it could be concluded that there is a consistent positive relationship between smoking and NSOFC. Therefore, avoidance of smoking to reduce this risk is a common public health message (World Health Organization, 2013).

In Saudi Arabia, the median prevalence of smoking in adults is 22.6%, which is considered high compared to global figures. However, in females alone the prevalence is low (9%) (Bassiony, 2009). A higher figure was reported by Fida and Abdelmoneim (2013), reaching 37% among males ranging from 16 to 22 years old attending secondary schools. The main types of smoking utilized were tobacco and waterpipe. Previous research has found that waterpipe smokers have significantly higher carboxyhaemoglobin concentrations than do cigaratte smokers; have higher carbon monoxide levels and also higher serum cotinine levels due to the larger amount of tobacco consumed during sheesha smoking compared with cigarette smoking (Zahran et al., 1985; Ardawi et al., 2007;). In addition, Shihadeh (2003) in his study to elucidate the mainstream smoke aerosol and thermal-fluid processes of the waterpipe, has suggested that smoking a single waterpipe session produces as much "tar" as 20 cigarettes. A systematic review on the effects of waterpipe smoking on health outcomes was carried out in 2010 and concluded that there is a significant association between waterpipe smoking and multiple adverse health outcomes (Akl et al., 2010). However, the relationship between these different smoking formats and NSOFC has yet to be established.

2.8.6.3 Alcohol consumption:

Alcohol consumption during pregnancy and the pregestation period has been associated with multiple birth defects, the constellation defining Foetal Alcohol Syndrome (FAS) or other alcohol related birth defects (ORBD) and neurotoxicity (Committee to study Foetal Alcohol Syndrome, 1996). Cartwright and Smith (1995) reported increased cell death in embryos, especially of cranial neural crest cells with ethanol exposure. They proposed that this could lead to birth defects following maternal alcohol consumption. Consistent with this, a cohort population based study that included 4714 woman and associated prenatal alcohol consumption with birth defects found a significant, four-fold increase in birth defects (OR:4.6 and 95% CI: 1.5 to 14.3) (O'Leary et al., 2010).

Alcohol consumption has also been considered a risk factor in the aetiology of NSOFC. It could be directly associated with OFC or could act as a confounding factor for other risk variables such as SES and maternal stress (Mossey, 2001a; Carmichael et al., 2007b). Werler et al. (1991) carried out a case-control study on 1464 infants to assess the relationship between alcohol consumption and selected birth defects including CL/P, and reported an increased risk of CL/P with severe alcohol consumption (five or more drinks per day) (OR: 3.0 and 95 % CI: 1.1 to 8.5). In addition, in a hospital-based case-control study including 274 cases and 548 controls, Leite and Koifman (2009) reported an increased NSOFC risk with maternal alcohol consumption during the 1st trimester (for CL/P: OR: 2.08 and 95% CI: 1.27 to 3.41, and for CP: OR: 2.89 and 95% CI: 1.25 to 8.30). They also found an increased NSOFC risk with increased dosage of maternal alcohol consumption (Leite and Koifman, 2009).

Molina-Solana et al. (2013) carried out a meta-analysis on other five case-control studies investigating the relationship between alcohol and CL/P and also reported an

increased risk of CL/P with maternal alcohol consumption (OR: 1.5 and 95% CI: 0.99 to 1.66).

A systematic review that assesses the relationship between parental alcohol consumption and NSOFC is still needed. Studies that confirm dose related effect of alcohol is also required.

2.8.7 Maternal stress

Studies have reported a relationship between maternal stress and NSOFC (Carmichael and Shaw, 2000; Laumon et al., 1996; Czeizel and Nagy, 1986; Saxen, 1974). Measurement of maternal stress is carried out through identification and scoring of stressful maternal life events including family problems, difficult pregnancies and occupational stress. Most studies have only incorporated one or two of these life events (Carmichael et al., 2007b).

2.8.7.1 Family problems and other maternal stressful events:

The effect of maternal stress on NSOFC could be related to elevated maternal corticotrophin-releasing hormone and corticosteroid levels resulting from stressful-events (Montenegro et al., 1995; Hobel et al., 1999), suggested by findings that corticosteroid medication can increase the risk of NSOFC (Carmichael et al., 2007a). Another explanation of how stress might cause birth defects is through negative coping behaviours that might be associated with stress, such as smoking (Baker et al., 2002; Azagba and Sharaf, 2011).

In Chile, Montenegro et al. (1995) measured the prevalence of NSOFC in mothers exposed to a severe shock from a large earthquake in 1985, compared to the prevalence of NSOFC in previous years. A significant increase was identified in the prevalence of NSOFC following severe shock, from 1.6/1000 births to 2.01/1000 births. To further strengthen their hypothesis they conducted similar stress intensity tests on pregnant mice and found a similar effect

(Angelica Montenegro et al., 1995). Furthermore, in a meta-analysis carried out on 28 case-control studies, Molina-Solana et al. (2013) analysed two studies assessing the relationship between stress and CL/P and found a significant relationship as well (OR:1.41 and 95% CI: 1.2 to 1.65). In addition, Radojicic et al. (2007) carried out a study on 96 OFC infants and their mothers compared to 122 controls in Belgrade after war to measure the effect of stress on OFC prevalence. Stress, anxiety, and disorganized emotional life were greater in cases compares to controls,

Further cohort longitudinal studies and studies that measures stress biomarkers are required to verify the relationship between maternal stress and NSOFC.

2.8.7.2 Hyperemesis gravidarum:

Hyperemesis gravidarum, a severe maternal nausea and vomiting, which accompanies pregnancy (usually in the 1st trimester), has been suggested as having a negative association decreasing NSOFC risk. A reduced chance of having an infant with NSOFC was reported to be associated with nausea and vomiting in the HCCSCA study to be (Czeizel et al., 1999). Krapels et al. (2006) reported that mothers with extreme vomiting were identified more frequently in a control population compared to in mothers of cases (for CL/P, OR:0.8 and 95% CI: 0.5 to 1.3; for CP, OR: 0.9 and 95% CI: 0.5 to 2). One hypothesis behind the suggested protective effect is that initiation of nausea and palatal fusion processes are related and are both positively affected by the changes in hormones and diet that accompany maternal vomiting. Another explanation could be related to the medication ingested to treat hyperemesis gravidarum. On the other hand, Czeizel and Vargha (2003) reported a greater ingestion of thiethylperazine in the treatment of severe vomiting in maternal cases with CL/P than in controls in his Hungarian population based case-control study (OR: 2.0 and 95% CI: 1.0 to 4.0). They also reported in 2005, no significant differences between mothers of infants

with congenital anomalies using dimenhydrinate which is an anti-emetic drug, and mothers with normal infants (OR: 0.9 and 95% CI: 0.8 to 1.0) (Czeizel and Vargha, 2005). In 2004, a prospective cohort-control study was carried out to compare pregnancy outcomes in women not experiencing nausea and vomiting during pregnancy with those experiencing nausea and vomiting at two levels of clinical severity. No association was found between lack of nausea and vomiting during pregnancy and an increase in the overall rates of major malformations (Boskovic et al., 2004). In addition, a Chinese case-control study reported a significant relationship between maternal nausea or vomiting during pregnancy and CP (OR: 1.82, 95% CI: 1.21 to 3.13) (Jia et al., 2011).

The relationship between nausea and vomiting during pregnancy and NSOFC is complex and needs further investigation to clarify.

2.8.8 Maternal domestic environmental exposure

Maternal domestic exposure could be from different sources. Pregnant women could be exposed to chemicals while she is at home or during her work. The following section discusses some of these exposures.

2.8.8.1 Maternal exposure to chemicals:

Studies have reported an increased risk of NSOFC after maternal exposure to chemicals such as; aromatic solvents, chlorinated solvents and Stoddard solvent (Desrosiers et al., 2012); organic solvents, lead compounds, and non-ionising and ionizing radiations (Chevrier et al., 2006); aromatic solvent and Stoddard (Garlantézec et al., 2009)

Testud et al. (2010) conducted a prospective cohort study on 206 mothers exposed to organic solvents using bio-monitoring for risk assessment and found no increased adverse outcome. In 2007, Romitti et al. conducted a meta-analysis on maternal exposure to pesticides and its association to NSOFC. Their results suggested that maternal exposure to pesticides is associated with a modest but marginally significant risk of clefting (Romitti et al., 2007). Further studies are required to identify maternal chemical exposure effects and its interaction with genes in the aetiology of NSOFC phenotypes. .

2.8.8.2 Occupational exposures:

Occupational exposure has been suggested to play a role in the aetiology of NSOFC. Desrosiers et al. (2012) found no significant relationship between occupational exposure to organic solvents chlorinated solvents during early pregnancy and risks of neural tube defects and orofacial clefts (OR:1.32 and 95% CI: 0.77 to 2.29). Langlois et al. (2013) carried out a population-based case-control study in the United States to investigate the relationship between polycyclic aromatic hydrocarbons and NSOFC. Occupational exposure was assessed by two to three industrial hygienists. The study included 805 CL/P and 439 CP cases compared to 2989 controls. There was a significant increase in the incidence of CL/P with maternal occupational exposure (OR: 1.69 and 95% CI: 1.18 to 2.40) but not of CP (Latchman, 1997). Qi et al. (2013) carried out a hospital based case-control study in Hubei Province, China that included 180 CL/P cases matched by age and sex with 108 controls. Paternal occupational exposure including noise, gasoline, radiation, and high temperature was significantly associated with CL/P (OR: 13.08 and 95% CI: 2.35 to 72.86).

Occupational exposures still need further investigation in cohort longitudinal studies. Also, genetic-occupational exposure interactions should be investigated to clarify their association with NSOFC.

2.8.8.3 Environmental contamination:

Dolk et al. (1998) carried out a multi-centre case-control study that assessed the risk of congenital anomalies associated with maternal residence near hazardous-waste landfill sites in Europe. An increased risk of congenital anomalies was observed for babies with mothers living within 3 km of hazardous chemical waste landfills (OR: 1.33 and 95% CI: 1.11 to 1.59). However, although there was an increase in the OR for OFC, the relationship was not significant (CL/P: OR: 1.18 and 95% CI: 0.66 to 2.12; and CP: OR: 1.63 and 95% CI: 0.77 to 3.41). The OR reported by the author was adjusted for SES and maternal age.

Ramakrishnan et al. (2013) conducted a case-control study assessing the association between estimated maternal residential exposure to benzene, toluene, ethyl benzene, and xylene (BTEX) and the risk of NSOFC. Agents were selected using the U.S. Environmental Protection Agency 2005 Hazardous Air Pollutant Exposure Model (HAPEM5). The study included 6045 NSOFC cases (3915 CL/P and 2130 CP individuals born between 1999 and 2008), with 6000 age-matched controls. Maternal residential exposure to benzene was not associated with oral clefts (OR: 0.95 and 95% CI: 0.81 to 1.12 for CL/P; and OR: 0.85 and 95% CI: 0.67 to 1.09 for CP).

Radiation contamination has also been considered in the aetiology of birth defects in a study by Mangones et al. (2013) in New York. Four zones of 5-mile increments were used to categorize proximity to the Indian Point nuclear reactor for individuals identified by analysing New York State Vital Statistics and Congenital Malformations Registry data (from 1992 to 2001). Over the 10-year period, 328,124 infants were born with 702 malformations yielding a regional rate of 2.1 major malformations per 1000 births. This prevalence is lower than the total malformation rate in New York (5.9/ 1000 births). Therefore, the prevalence of birth defects was not related to proximity to the nuclear power plant (Mangones et al., 2013).

From these studies we can conclude that although different environmental contamination exposures such as; radiation, hazardous-waste and chemicals, seems to have a relationship with NSOFC, further investigation to confirm or reject this relationship is still needed.

2.8.9 Family history and parental consanguinity

Family history and parental consanguineous marriages were reported to be associated with NSOFC. In the following section we will discuss this association

2.8.9.1 Family history

Family history of NSOFC has been reported to have a significant association with NSOFC increasing the chance of having a child with clefting (Jia et al., 2011). Lie et al. (1994) carried out a population based study that investigated the risk of birth defect recurrence among Norway woman who delivered their first and second children between 1967 and 1989. Clefting was considered to have a higher recurrence risk in the second infant (CL relative risk was 31.4 with 95% CI: 19 to 52 and CP relative risk was 44.5 with 95% CI: 9 to 134) compared to other birth defects with relative risk 7.6 and CI: 6.5 to 8.8. Zandi and Heidari (2011) reported a significant relationship between the presence of clefting in siblings or parents and having a child with OFC. Furthermore, in a case-control study in the Netherlands, Krapels et al. (2005) reported that the most significant variable related to NSOFC was family history (OR: 19, CI: 1.1 to 2).

2.8.9.2 Parental consanguinity

Consanguinity has been reported to be higher in parents of children with orofacial clefts than in those without (Zeiton et al., 1993; Zlotogora, 1997; Elahi et al., 2004; Reddy et al., 2010). It has been suggested that consanguinity could play a role in the aetiology of clefts. Saudi

Arabia has a high prevalence of consanguinity (57.7%) (El-Hazmi et al., 1995) and presents an opportunity to carry out such an investigation. In King Faisal Hospital and Research Centre, it was found that 28% of patients with clefting were first cousins, rather a high rate when compared to the prevalence of 1st cousin consanguineous marriages reported in the general Saudi population (Al-Johar et al., 2009). To clarify the relationship between consanguinity and NSOFC, a systematic review and meta-analysis has been carried out for all case-control studies that have measured the relationship between consanguinity and NSOFC, concluding that consanguinity is indeed a risk factor for NSOFC (OR: 1.83 and 95% CI: 1.31 to 2.54) (Sabbagh et al., 2014) (appendix B3). One of the studies included in the systematic review was carried out in Saudi Arabia and reported an association between orofacial clefts and consanguinity (Alsahafi, 2010).

2.8.9.3 Family history with parental consanguinity

The presence of consanguineous marriages in a community has been suggested to increase the prevalence of congenital anomalies related to recessive gene disorders (Pritchard and Korf, 2008). Moreover, in consanguineous marriages where there is known family histories of birth anomalies, the chances of inheritance and occurrence of defects are expected to increase further (Saggar and Bittles, 2008). In a Saudi Arabian study, Al-johar (2008) reported positive family history in almost a quarter of the cleft patients with related parents. Moreover, in Saudi Arabia, Ravichandran et al. (2012) found a statistically significant difference between consanguineous and non-consanguineous marriages in relation to family history of clefting. Further investigations confirming the relationship between consanguinity and family history and their relationship to NSOFC are needed, as well as to identify the risk potential in these families and to provide suitable information for prenatal and premarital genetic consultation.

2.9 Gene-environmental interaction

Gene-environmental interactions (GEI) have been suggested to play an important role in the aetiology of NSOFC. It is defined biologically as the coparticipation of the genetic and environmental risk factors in the same causal mechanism to promote disease development (Zhu et al. 2009). One of the important applications of GEI studies is to help public health researchers develop strategies for targeted intervention for risk-factor modification based on individual genetic profile (Hunter 2005).

The most common study designs used to assess GEI include: population-based cohort study, case-control study, case-only study and family-based design. The advantage, disadvantage and indication were discussed in several papers (Umbach and Weinberg 2000; Zhu et al. 2009; Hutter et al. 2013). Case-control studies tend to be more feasible and cost efficient than cohort studies. However, they suffer from the possibility of stratification bias. Case-only studies has the advantage of being simple, do not need controls, and are more efficient than case-controls when the main goal is studying gene-environmental interaction and distribution of the different exposures according to their genotypes. However, they do not measure the joint effect of gene and environmental exposures in relationship to the aetiology of the disease. Family-based designs have the advantage of overcoming confounding due to population stratification. However, error can arise when the variant allele affects the disease risk or where the test considers that transmission from heterozygous parents as independent events when they are actually not (Umbach and Weinberg, 2000).

2.9.1 Maternal supplementation-gene interaction

The effects of folic acid supplementation and its interaction with genes have been investigated in multiple studies. For NSOFC, it has been found that the unfavourable effects of low folate intake on the risk of developing an OFC infant were more pronounced in mothers with MTHFR 677TT or MTHFR 1298CC genotypes (Van Rooij et al., 2003b).

In 2008, Boyles et al. studied the association between twelve polymorphisms in nine genes related to one-carbon metabolism, and clefts in a Norwegian population-based study that including 362 CL/P and 191 CP infant-parental triads. They stratified maternal periconception intake of folic acid to investigate gene-supplementation interactions, and reported a reduced risk of CL/P in mothers who carried the *CBS* C699T variant (rs234706) (Czeizel and Rockenbauer, 1998; Soprano and Soprano, 1995) (P= 0.008, relative risk (RR): 0.50 and 95% CI: 0.26 to 0.96). However, they found no evidence of significant interaction between the *MTHFR* C677T variant (rs1801133) and maternal folate intake (Boyles et al., 2008).

In 2009, Boyles et al. further investigated the effects of genes involved in folate and vitamin A metabolism on the occurrence of NSOFC using pathway associated study. They analysed 29 genes involved in folate /one carbon metabolism and 16 genes involved in vitamin A metabolism. Their analysis, stratified for maternal intake of both folate and Vitamin A, found that the strongest association identified was between foetal *FOLH1* and CP only (Boyles et al., 2009). Further evidence for interaction is provided by Velázquez-Aragón et al. (2012), who investigated the association and interaction between IRF6 (rs2235371 and rs2235375), 8q24 and maternal folic acid intake in a case-control study. Using logistic regression analysis, they found a statistically significant relationship between maternal folic acid intake and the 8q24 SNP rs17241253 in NSOFC infants.

In addition, Wu et al. (2010) carried out family-based design in China that aims to investigate the interaction between 21 SNPs on IRF6 and maternal multivitamins supplementations among CL/P cases. A significant interaction was found with IRF6 rs2076153 (P= 0.019) and rs17015218 (P= 0.012).

Further investigation is needed to clarify the interaction between folic acid and genes in the aetiology of NSOFC. In addition, the effect of maternal supplementation (multivitamins and folic acid) and their doses should be considered.

2.9.2 Maternal smoking-gene interaction

Gene-environment interaction has also been observed between maternal exposure to smoking and the ability of the mother and fetal enzymes to detoxify the toxic compounds of smoking as demonstrated from studies that examined genes involved in the detoxification pathway (Shi et al., 2008). Krapels (2005) identified roles for polymorphisms in *EPHX* exons 3 and 4 and in *GSTPI* exon 5, as well as an interaction between parental *EPHX* exon 4 and periconception smoking in the aetiology of OFC.

In 2008, Shi et al. carried out a review of studies investigating interaction between genetic and maternal smoking in contributing to oral cleft. They comprised 19 studies with different methodologies and results. Genes that were investigated includes: TGFA, MSX1, BCL3, MTHFR GSTP1, GSTT1, GSTM1, GSTM3, HIF1A, NAT2, EPHX1, UGT1A7, GST, AhR, TGFA, NOS3, RARA. Studies were either case-control or case-triad. The interaction between genetic variation at the *TGFA* locus and maternal smoking in the aetiology of OFC was investigated by Shaw et al. (1996), who found that the risk of CL/P increased from three to eleven-fold for infants with rare *TGFA* allele when mothers smoked more than 20

cigarettes per day. Lammer et al. (2004) have found that infants with a *NAT1* polymorphism are at increased risk of NSOFC in mothers who smoke. In addition, Shi et al. (2007) found a significant interaction between maternal smoking, the *UGT1A7* and GSTT1 null-gene in mother and the occurrence of NSOFC in a case-control study (Shi et al., 2007). Zeiger et al. (2005) found a significant joint effect along with interaction between maternal smoking and *TGFA* for CP only. Shi et al (2008) review concluded that GEI studies are still in their early stage.

Further GEI was carried out by Chevrier et al. (2008) which did not confirm the relationship or interaction between maternal smoking and *GSTT1* in NSOFC children. Recently, Wu et al. (2010) reported a significant relationship between maternal passive smoking and rs1044516 (P= 0.041) using condition logistic regression and family based association test.

The interaction between genes and smoking is still in its preliminary stage. There are many factors that need to be considered in future smoking-gene interaction NSOFC research. For example; the fact that NSOFC is a multifactorial birth defect with many genes to be investigated. In addition, researcher needs to consider smoking intensity levels and the type of smoking devices.

2.10 Gene-gene interaction

Blanton (2010) found a significant interaction between *IRF6* (rs642961) and three SNPs located in 8q24 (rs1530300, rs17241253, and rs987525) in a non-Hispanic white population with NSOFC. In addition, in an Asian cohort, Butali et al. (2013) found statistically significant interactions between *MAFB* rs17820943 and *PAX7* rs4920520 (P= 0.03), *VAX1* rs7078160 (P= 0.05) and *VAX1* rs4752028 (P= 0.03); and between *VAX1* rs7078160 and *FOXE1* rs3758249 (P= 0.01). Moreover, there was evidence for interaction in a combined

sample across all populations between *VAX1* rs4752028 and both 8q24 rs987525 (P= 0.03) and *MAFB* rs17820943 (P= 0.05).

2.11 Review summary

The aetiology of NSOFC is still unclear and needs further research and analysis. The literature reviled several important environmental factors such as; smoking, dietary factors and supplementations, medications and illness, and maternal exposure to chemicals. However, most of these factors still need additional confirmation from future studies. For genetic factors, the only gene consistently found to be associated with oral cleft is IRF6. However, the aetiology of oral cleft varies geographically. Therefore, it still needs to be studied in Saudi Arabia

2.12 Rationale for candidate gene selection

2.12.1 Interferon regulatory factor 6 (*IRF6*)

- *IRF6* is the only gene to have shown consistently significant results across studies.
- Results for *IRF6* and OFC association reached genome wide significance.
- *IRF6* has shown genetic interaction with the locus (8q24) demonstrating the most significant association to NSOFC.

2.12.2. Ventral Anterior Homeobox (*VAX1*)

- Homozygosity of a rare variant of *VAX1* has been found in a patient with consanguineous parents and NSOFC.

- Saudi Arabia has a high prevalence of consanguinity, suggesting the potential for high efficiency in detecting recessive genetic effects.

- The locus containing *VAX1*, 10q25 has reached genome wide significance in NSOFC association studies.

- There is a new evidence for significant association between *VAX1* and NSOFC in the literature exposing an under-researched area.

Chapter 3: Aims and objectives

3.1 Aims

To investigate the birth prevalence, environmental risk factors, genetic risk factors, and gene-environmental interactions associated with non-syndromic orofacial cleft (NSOFC) in the Central and Western Region of Saudi Arabia.

3.2. Objectives

3.2.1 Prevalence of NSOFC and the influence of paternal consanguinity among CL/P and CP in Saudi Arabia

1. Compare and describe the birth prevalence and other epidemiological characteristics of NSOFC in the Central and Western Region of Saudi Arabia.
2. Compare and describe the birth prevalence of CL/P and CP in the Central and Western Region of Saudi Arabia.
3. Investigate the prevalence of paternal consanguinity among different NSOFC sub-phenotypes and their severity.

3.2.2 Environmental risk factors associated with CL/P and CP in Saudi Arabia

4. Investigate the relationship between different demographic variables and NSOFC phenotypes (CL/P and CP).
5. Investigate the relationship between socioeconomic status (SES) and NSOFC phenotypes (CL/P and CP).
6. Investigate the relationship between family history and NSOFC phenotypes (CL/P and CP).

7. Investigate the relationship between maternal illnesses, medications, and multivitamin supplementation three months prior to pregnancy and in the 1st trimester period, and having offspring with CL/P or CP.
8. Investigate the relationship between the different forms of parental smoking and having an infant with CL/P or CP.
9. Investigate the relationship between maternal chemical exposure and maternal drinking water source type three months prior to pregnancy and in the 1st trimester period and having a child with CL/P or CP.

3.2.3 Genetic risk factors associated with CL/P and CP

10. Investigate the association between the infant-parental *IRF6* gene polymorphisms and the parent of origin effect with regard to NSOFC and its phenotypes (CL/P and CP) in the Saudi population.
11. Investigate the association between infant-parental *VAX1* gene polymorphisms and the parent of origin effect with regard to NSOFC and its phenotypes (CL/P and CP) in the Saudi population.
12. Investigate the relationship between *IRF6* and *VAX1* haplotypes and NSOFC occurrence among infants in Saudi Arabia.
13. Investigate the relationship between parental consanguinity and infants' *VAX1* polymorphisms in the risk of NSOFC and its phenotypes (CL/P and CP) among infants in Saudi Arabia.

3.2.4 Gene-environmental interactions associated with NSOFC in Saudi Arabia

14. Investigate the role of maternal gene-environmental interactions in the aetiology of orofacial clefts.

3.3 Null hypotheses

The following null hypotheses will be tested

3.3.1 Prevalence of NSOFC and the influence of paternal consanguinity on CL/P and CP in Saudi Arabia

1. There is no difference between the prevalence of CL/P and CP in Saudi Arabia and global figures.
2. Parental consanguinity does not influence the prevalence of CL/P and CP in Saudi Arabia.

3.3.2 Environmental variables

3. There is no difference between cases and controls in their epidemiological variables.
4. There is no difference between cases and controls in their socioeconomic status.
5. Maternal medication, illness, fever or dietary supplementation in the pre-gestation and 1st trimester period do not influence the risk of having an infant with CL/P or CP in Saudi Arabia.
6. There is no difference between cases and controls in the frequency of mothers experiencing stress during the pre-gestation and 1st trimester periods.

7. Maternal exposures to smoking, chemicals and paternal smoking do not increase the risk of having an infant with CL/P or CP in Saudi Arabia.

8. The source or type of drinking water for pregnant woman does not play a role in NSOFC occurrence in Saudi Arabia.

3.3.3 Genetic factors

9. In an infant-parental triad study design, *IRF6* and *VAX1* polymorphic variants, transmission and frequency do not play a role in the occurrence of NSOFC (CL/P or CP) among infants in Saudi Arabia.

10. *IRF6* and *VAX1* haplotypes are not risk factors in the occurrence of NSOFC among infants in Saudi Arabia.

11. Parental consanguinity does not increase the risk of NSOFC in infants with *VAX1* rare alleles in Saudi Arabia.

3.3.4 Gene-environment interactions

12. The interaction between maternal environmental exposures during the pre-gestation and 1st trimester periods and maternal *IRF6* and *VAX1* polymorphic variants does not play a role in the aetiology of NSOFC among infants in Saudi Arabia.

CHAPTER 4: Materials and Methods

4.1 Study design

4.1.1. Study design (Part I)

The case-control study was preceded by a retrospective descriptive study to measure the birth prevalence and characteristics of NSOFC in the Western and Central regions of Saudi Arabia, and to examine the influence of consanguinity on these statistics.

4.1.2 Study design (Part II)

A case-control study to investigate the environmental and genetic risk factors related to NSOFC using parent-child triads and matched controls in the Central and Western regions of Saudi Arabia was planned. One of the requirements of the Federal government for establishment of the study was to carry out the genetic analysis within Saudi Arabia. Therefore, the research was coordinated and funded by King Abdulaziz University in Jeddah, Saudi Arabia and the genetic analyses were carried out in the Princess Al-Johara Hereditary Centre.

4.2 Study setting (Part I and II)

Saudi Arabia is divided to three regions: Central, Western, and Eastern. The Western region comprises one third of the Saudi population and is further divided into Makkah and Maddina regions. The Central region consists mainly of the Riyadh region and comprises one-third the population (Ministry of Health, 2010). Cities with the highest population in each area were selected for this study (Riyadh, Jeddah, and Maddina), encompassing almost 50% of the Saudi population (Ministry of Health, 2010; Central Department of Statistics and

Information, 2013) (Figure 6). Each city was divided in to five districts (central, north, south, west and east), and from each district, the hospital with the highest number of referrals and births was selected (Table 4.1). These hospitals were governmental hospitals and were defined by the Ministry of Health (MOH) (2006) to include Ministry of Health hospitals, University hospitals, National Guard Hospitals, and the King Faisal Specialized Hospital and Research Centre. According to Almalki et al. (2011) the governmental hospitals delivers health care services to 79% of the Saudi population in Saudi Arabia.

4.2.1 Subjects

4.2.1.1. Subjects (Part I):

Part I of the study included all infants born in the designated hospitals between the 1st of January 2010 and the 1st of January 2012. Hospitals that were excluded from part I were: the King Fahad Hospital in Jeddah as it is a trauma centres and do not have delivery units; and the Riyadh Military Hospital as it did not approve this part of the research because it has similar ongoing research.

In Jeddah, the Al-Messadia Maternity Hospital and the Al-Azizia Maternity Hospital account for 67.3% of the total births in the Ministry of Health hospitals (Ministry of Health, 2010). In addition, Maternity and Children's Hospital is the only governmental maternity hospital and referral centre for OFC in Maddina.

4.2.1.2 Subjects (Part II):

Part II included infants with NSOFC, and their parents, born at or referred to the selected hospitals from January 2010 to January 2012. The age of inclusion for children ranged from 0 to 18 months. These child-parent triads were clustered under the ‘study group’.

The ‘control group’ consisted of infants without clefts born at the hospital, or attending for vaccination or trauma. The control group was matched with the study group for age, gender, and hospital where they were seen. Ethnicity was not included in the matching criteria.

Figure 4.1**:** Map of Saudi Arabia showing the cities that were included in the study (stars) and their associated regions.

Table 4.1: List of medical centres included in the study according to their regional location in each city.

City	Center	North	South	West	East
Jeddah	Maternity Hospital Almesadiah & King Fahad Hospital (KFH)	King Abdulaziz University Hospital (KAUH)	King Abdulaziz Medical City (KAMC)	King Fahad Armed Hospital (KFAH)	Maternity Hospital Alaziziah
Riyadh	King Fahad Medical City (KFMC)	King Faisal Specialized Hospital and Research Centre (KFSHRC)	King Saud Medical City Hospital	Riyadh Military Hospital (RMH)	Riyadh National Guard Hospital (RNGH)
Maddina	Maddina Maternity and Children Hospital				

4.2.2 Sample size prediction:

- Preliminary sample size was predicted from the total births recorded for the Western and Central regions of Saudi Arabia as described in the statistical book of the MOH (2006) at 74,000 births/year. Comparing this figure with the global NSOFC birth prevalence (1.25/1000 births), it was expected that 185 infants would be born with

NSOFC over a 24 month period (Ministry of Health, 2006). However, when the prevalence was calculated for the total births reported by MOH in 2010 (98,000 births/year), the number of NSOFC patients expected was 245 infants over two years.

- The second approach to predicting sample size was through writing a systematic review for the prevalence of NSOFC in Saudi Arabia and Middle Eastern countries that have similar and comparable population characteristics (Sabbagh et al., 2012) (Appendix B1). Only eight articles were cited. Four studies were carried out in two regions in Saudi Arabia (Riyadh and Al-Qaseem). The prevalence of NSOFC reported varied greatly from 0.3 to 2.4 per 1000 live births with a mean prevalence of 1.25 per 1000 births. The total births per year in the three cities included in the study were about 98,000, according to the MOH (2010) (Ministry of Health, 2010). Therefore, the expected number of NSOFC births in 24 months was calculated to be 245 patients.

- The third approach used to predict the sample size was through retrospective measurement of the prevalence of NSOFC in Jeddah in the year 2009. This was carried out by checking the medical records of the five hospitals selected in Jeddah. There were 15 non-syndromic cleft lip and/or palate living births born in 2010. This number was compared with the total number of live-births in 2010 at the same hospitals (23,970). Accordingly, the prevalence of cleft lip and/or palate in Jeddah was calculated to be 0.62 for every 1000 live-births. This leads to an estimated sample size much smaller than that calculated using the first approach. If we consider the other cities to have the same birth prevalence, the estimated number of patients with NSOFC in 24 months would only be 121 patients.

- Finally, the sample size was measured using Open Source Epidemiologic Statistics for Public Health (OpenEpi) online software for epidemiologic statistics:
    - For part I (NSOFC prevalence) the sample size was measured using a 95% confidence interval and using the global prevalence figure (1.25/1000), yielding a recommended the sample size of 61,055 live-births.
    - For part II (case-control study) the sample size was measured using values of a 20% expected control exposure, an 80% power (the study has an 80% chance of ending up with a p value of less than 5%) and an OR of 2. The expected exposures and OR used were suggested according to the previous literature reports. By convention, the 80% statistical power is considered the standard and an acceptable power. This resulted in a sample size of 187 cases and 187 controls. When the OR used was 2.5 the sample size was 105 cases.

      http://www.openepi.com/oe2.3/menu/openepimenu.htm.

Therefore, on the basis of this sample size calculation and expecting 60% participation, it was decided that for part II case-control analysis a reasonable power could be achieved by recruiting a study sample of between 105 and 187 patients and a similar number of controls over a 24 month period.

4.2.3 Methods

4.2.3.1 Ethical approval:

The following were submitted to each Institutional Research Review Board (IRB) in every research centre and medical health institution (Appendix A3 to A9):

- A research proposal describing the research purpose, materials and methods, projected outcome and patients' expectations from the research.
- The required request for research approval papers for each hospital and for the Ministry of Health were completed, signed by the Head of Preventive Dental Sciences Department in King Abdulaziz University, and attached to the research proposal.
- A principle investigator (PI) working in each of the included hospitals was named.
- Patient consent form that included a summary on the project, the procedure, what is expected from the patient, what is expected from the research, patient confidentiality, if any side effects exist and a space for patient signature.

All of this information was sent with the ethical approval request (in both English and Arabic), along with the name of the person assigned to collect the data, to each IRB committee for approval.

The dates for requesting and receipt of approval are listed in Appendices A10.

4.2.3.2 Patients' Consent form:

A consent form was prepared in Arabic and given to parents for signature after they were provided with verbal and written information concerning what, why and how the research was to be carried out. The form also included information on the side effects, confidentiality, freedom of participation, and what the family should expect from the research (see Appendix A4 and A8). Families were given 50 Saudi Riyals as a payment to compensate for their transportation and additional time. The consent form was signed prior to examination of the infant and was checked by the nurses in the unit as required by the Ministry of Health.

4.2.3.3 Principal Investigator (PI):

All hospitals, except the Ministry of Heath, required a PI to receive research approval. In Jeddah, the PI was not directly involved in the research. However, in Riyadh and Maddina they were involved in data collection. The PIs were chosen either because they were interested in the research or were recommended by the institute. These PIs were our research collaborators and are listed in Table 4.2.

Table 4.2: List of principal investigators (PI) that were chosen as a requirement for the research approval request for each medical centre in each city.

City	Hospital	Name of PI
Jeddah	KAUH	Prof. Najlaa Alamoudi
	NGH	Dr. Mamoon Daghistani
	KFH	Dr. Hassan Alamary
Riyadh	KFMC	Dr. Sari Rabah
	KFSHRC	Dr. Azizah Al-Johar
	KAMC	Dr. Nasir Alhamlan
	KFH	Dr. Mustafa Hamdan
	RMH	Dr. Eman Alnamnakani
Maddina	Maternity and Children Hospital	Dr. Fatma Abdulhameed

4.2.3.4 Ascertainment:

In order to ensure that the best possible standards were utilized for ascertainment, procedures were followed according to the recommendations of the Global Registry and Database on Craniofacial Anomalies (2003) (World Health Organization, 2003). These procedures were adapted to suit local practice without allowing consistency and rigor to be negatively impacted as follows:

4.2.3.4.1 Number of examiners:

The least possible number of examiners were selected in each city for recruitment of cases and controls. In Jeddah, all data were gathered by a single person, who was the main research coordinator for the Saudi-cleft project. In Maddina, there a single plastic surgeon was responsible for collecting the data. However, in Riyadh four examiners were trained for data collection: two were plastic surgeons and two were orthodontists.

4.2.3.4.2. Examiner training:

The examiners were trained at a formal meeting that provided detailed explanations regarding the questionnaire and the questionnaire coding manual (see Appendix A9). Furthermore, examiners were trained on how to use Oragene kits for saliva collection from parents and infants and how to store collected samples.

4.2.3.4.3. Flexibility of the research protocol:

The protocol for data collection was modified according to the needs and circumstances of each institution. It was distributed in files and listed under the institution name and code. A copy of the protocol was given to each research coordinator. Protocols were designed specifically for each hospital's requirements. For example, the details of the storage and travelling protocols for hospitals in Riyadh and Maddina were modified respectively.

4.2.3.4.4 Illustrative material to ensure clarity of the research protocol:

A file containing complete information on the project including the summary of the research; proposal; materials and methods; flow chart, patients' coding manual, and questionnaire is provided (see Appendix A3 to A9).

4.2.3.4.5 Assurance of patient recruitment and sample size fulfilment:

For part I, the medical records were reviewed retrospectively every 6 months to ensure that all cases born in the designated hospitals were contacted.

4.2.3.5 Methods of sample recruitment:

4.2.3.5.1 Study group (part I and II):

The study group was recruited and ascertained in three ways:

- Nurses working in the neonatal unit, neonatal intensive care unit, plastic surgery and orthodontic clinics were instructed to contact the research coordinator immediately once a child with OFC was signed into their unit. Furthermore, the research coordinator inquired regarding new cases every two weeks.
- Records of the neonatal units since June 2009 were reviewed retrospectively for all infants born with non-syndromic OFC. These infants were contacted and given an appointment in the dental clinic for examination and sample collection.
- Every 6 months the neonatal unit records were reviewed to ensure that all cases born in the selected hospitals were examined. If not, missing cases were contacted.

4.2.3.5.2 Control group (part II):

In part II of the study, the control group was recruited from the same institutions in the same period of time as were the study group (from January 2010 to January 2012). Controls were recruited according to the following criteria:

- If an NSOFC infant was encountered immediately after birth, the next infant to be born in the same hospital with the same gender was selected as a matched control; or
- If the NSOFC infant was a referred case, a control matched for age and gender was recruited from primary health care clinics (vaccination clinics) or from infants

admitted to the pediatric unit for reasons other than birth defects or chronic disease (e.g., trauma or GIT infections).

4.2.3.5.3 Comparison group (part I):

To measure the NSOFC prevalence in part I of the research, the total births in the designated hospitals from the 1st of January 2010 to the 1st of January 2012 were retrieved from the statistical records of each hospital.

4.2.3.6. Clinical examination:

Clinical examination of NSOFC infants in parts I and II was carried out in pediatric neonatal unit, pediatric plastic surgery units or in orthodontic clinics using lights and mirrors to detect the type and characteristics of the clefts utilizing the International Clearinghouse for Birth Defects Monitoring System (ICBDMS 1991; ICBDMS 2001). The research coordinator in each institution relied upon the diagnosis of the clinical geneticist, if available, or pediatrician for syndrome exclusion following cleft examination. If the case was ascertained prior to surgery, a picture was taken to document the cleft phenotype. The NSOFC was classified according to the LASHAL classification system as follows (Kriens, 1989):

- LASHAL stands for Lip, Alveolus, Soft Palate, Hard Palate, Alveolus and Lip.
- When read with left sided letters, it indicates a right side cleft.
- When in UPPER CASE, it indicates a complete cleft.
- When in lower case, it indicates partial or incomplete clefting.

Submucosal cleft palate was excluded from the diagnoses because of poor ascertainment and therefore variable detection rates. In many instances it was overlooked by the nurses and upon subsequent diagnosis, the research team was not contacted.

4.2.3.7 Data collection:

4.2.3.7.1. Questionnaire (environmental factors in part I and part II):

The questionnaire was written in both Arabic and English on separate forms. The Arabic form was for parents to view while in the waiting area to be aware of the questionnaire's content. The research coordinator then interviewed the parents after the consent form was signed. It took approximately 15 minutes to fulfill these steps. The questionnaire included questions related to (see Appendix A5):

- general information, including: date of participation, name of the hospital, place of birth, residence, day of birth (Arabic or English), the, name of the patient and their parents, and contact numbers;

- demographic data and pregnancy history, including: maternal age at delivery, maternal weight/height, length of pregnancy, time interval between the NSOFC infant and prior sibling where applicable, prenatal visits, child birth order, neonatal length, neonatal weight, and neonatal head circumference;

- socioeconomic status: parents' educational level, mother's working status, type of occupation during pregnancy, family monthly income "less than 4000RS (threshold for Saudi Salary, https://online.hrdf.org.sa/FAQ/faq.html), 4000 to 10,000, more than 10,000" and residency;

- family history of parental consanguinity and type of consanguinity;

- maternal exposures prior to pregnancies and in the 1st trimester periods including: medication, illness, disease, supplementation, tobacco, and chemicals;

- maternal stress as estimated by relevant questions on stressful events for the mother that occurred prior to pregnancies and in the 1st trimester periods. The questionnaire included family problems, severe morning sickness, abdominal pain, and depression.

4.2.3.8 Sources of DNA for genetic analysis (part II):

Sources of DNA for genetic analysis included saliva or blood samples. Our rationale for choosing the source of DNA and methods for DNA extraction and storage is explained below.

4.2.3.8.1 Saliva samples:

Saliva samples were collected from infants and parents in both groups. The donor was instructed not to eat, drink, or smoke up to 30 minutes prior to providing the sample. For parents, OG-500 Oragene kits were used (see Appendix A6). Parents were asked to spit 2 ml of saliva into the collection tube of the kit in order to obtain 110 μg of purified DNA, according to the manufacturer's instructions. It took about 7 minutes to obtain the required amount.

For infants, the OG-575 Oragene Kit was used to collect 0.75 ml of saliva. This kit contained a sponge that was applied to the sublingual area in the infant's mouth to absorb saliva. The sponge was then was squeezed against the V-notch in the collection tube and reapplied as necessary (see Appendix A7). It could take from 15 to 40 minutes to collect a sufficient amount of saliva from infants depending on the circumstances. If the newborn infant was asleep a much longer collection period was needed. The collected saliva was expected to yield 17.3 μg of DNA according to the manufacturer's instructions.

Both kinds of samples were then at room temperature (less than 30°C) until they were sent for DNA extraction processing in the genetics laboratory, where they were stored at 4°C.

4.2.3.8.1 Blood samples:

A blood sample was taken only if the clefted infants were undergoing surgery under general anaesthesia and had an intravenous (IV) line. In this circumstance, it was considered more convenient to collect blood than to obtain saliva. The blood samples were collected in an EDTA anticoagulant tube and stored at temperatures less than 4°C until they were sent to the genetics laboratory for DNA extraction. If they were obtained from another city they were transported and stored in wet ice. In the genetics laboratory they were stored at 0°C.

4.2.3.9 Coding system:

Each questionnaire from the study and control groups had a code consisting of two parts with 6 digits as follows:

4.2.3.9.1 The questionnaire code:

- Centre code: consisted of two digits. The first digit indicated the name of the city; the second digit indicated the name of the hospital. The list of included medical centres is presented in Appendix A10.

- Family code: started with either a 1 (study group) or a 2 (control group). The remaining number indicated the serial number.

4.2.3.9.2 Sample collection tube code:

Each sample tube was coded with the same code as on the questionnaire label, with the addition of a letter identifying from which family member the sample was obtained:

- M: mother’s sample;
- F: father’s sample; and
- C: child’s sample.

4.2.3.9.3 Genetic laboratory coding:

DNA extracted from saliva or blood samples of cases and controls was labelled with new family codes serialized from 1 to 300 with addition of the family member code (M, F, and I [infant]). The code was matched with the data collection code in SPSS tables for identification.

4.2.3.10 Pilot study:

A pilot study was carried out to check the validity of the questionnaire. Ten patients were interviewed using the proposed questionnaire. Accordingly, some questions were rephrased or modified for the final version of the questionnaire.

In addition, three saliva samples were collected to check the quality and quantity of DNA extracted from saliva using the Oragene kits. The DNA extracted proved to be satisfactory in terms of yield and quality (genomic DNA were intact and appear as a compact, high-molecular-weight band with no low-molecular-weight smears in agarose gel electrophoresis).

4.2.3.11 Molecular genetic analysis:

Molecular genetic analysis included two main steps: DNA extraction and PCR. For *IRF6* the three SNPs included in this study were analysed using restriction-digestion PCR. For *VAX1*, real-time PCR was used for analysis of variant alleles.

4.2.3.11.1 DNA extraction:

DNA extraction was carried out using the QIAamp DNA Mini Kit (Catalogue # 51306; Qiagen, Canada) according to manufacturer's protocol with modification of the starting quantity saliva (500 µl instead of 200 µl). The detailed protocol is presented in Appendix A11.

The purity and quantity of extracted DNA was measured using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Delaware, USA). Estimation of the DNA concentration in different samples was carried out by measuring the optical density at 260 nm according to the following equation:

Conc. (µg/ml) = OD260 × 50 × dilution factor.

The DNA quality was also evaluated using agarose gel electrophoresis (1% in 1× TBE (Tris/Borate/EDTA) buffer stained with Sybr Safe DNA Gel Stain (Invitrogen, California USA)). The result showed good quality DNA bands (genomic DNA were intact and appear as a compact, high-molecular-weight band with no low-molecular-weight smears in agarose gel electrophoresis) (Figure 4.2). DNA passing quality control measures was subject to subsequent amplification by polymerase chain-reaction (PCR) and genotyping.

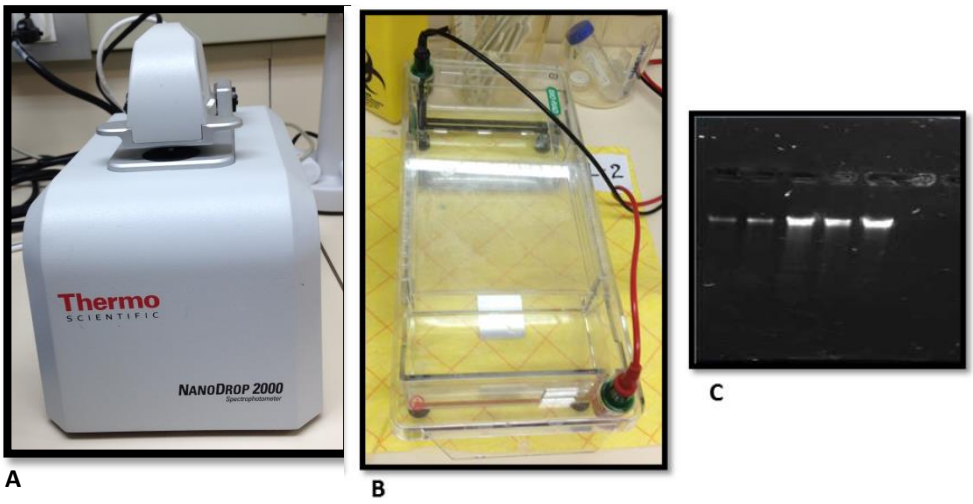


Figure 4.2: A) A Nanodrop 2000 spectrophotometer used to measure DNA purity and quantity. B) Gel electrophoresis apparatus (1% agarose in 1× TBE buffer) used to evaluate the quality of extracted DNA. C) Gel Electrophoresis image showing good quality bands of DNA extracted from saliva (genomic DNA were intact and appear as a compact, high-molecular-weight band with no low-molecular-weight smears) (analysis carried out by Edris and Al-Mahdi).

4.2.3.11.2 Genotyping for IRF6:

4.2.3.11.2 .1 Primer design:

Three primers pairs were designed to be specific for the three SNPs in *IRF6*. The primers designed for rs2013162 and rs2235375 were identical to those used by Huang et al. (2009), as was a universal M13 primer used for sequencing purposes. However, a new primer was designed for rs2235371 for this study using Primer Blast. Primers for *IRF6* SNPs are listed in Table 4.3.

Table 4.3: List of primers and amplification annealing temperature/melting condition used for *IRF6* SNP PCR.

***IRF6* SNP**	**Primers**	**Annealing temperature**
rs2013162	F: 5′- TGTAAAACGACGGCCAGTCCCTGGGAT GAGAAGGATAA - 3′ R: 5′- CAGGAAACAGCTATGACCACCTCTGACT CCCACTTGCT - 3′	57°C
rs2235375	F: 5′ - ACAGGAAAGAGTCTATAATAG AAGCAGAAGATC - 3′ R: 5′ - CCCAAAACTGAACCCCTGGAGAT - 3'	63°C
rs2235371	F: 5′ - TGTAAAACGACGGCCAGTCTCTGTCC ATGACGTCCAGC - 3′ R: 5′- CAGGAAACAGCTATGACCGAGTACGGG CAGACCATGAC - 3′	57°C

a. <u>PCR:</u>

The PCR amplification reaction was carried out in a 25-µl reaction volume using the GoTaq® Green Master Mix (Promega USA, Cat. #M7122) containing 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, and 2 units of Taq-DNA polymerase. Ten picomoles of each forward and reverse primers were added to the reaction mix along with 25 ng template DNA. PCR amplification was performed in a Veriti Applied Biosystems thermal cycler (Applied Biosysems, California USA) (Figure 4.3), programmed to complete 35 cycles after an initial denaturation cycle of 4 min at 94°C. Each amplification cycle consisted of a denaturation

step (94°C for 30 s), an annealing step (as indicated in Table 4.3 for 30 s), and an extension step (72°C for 30 s), followed by a final extension cycle (7 min at 72°C) in the final cycle.

Figure 4.3: Applied Biosystems (AB) machine used for PCR.

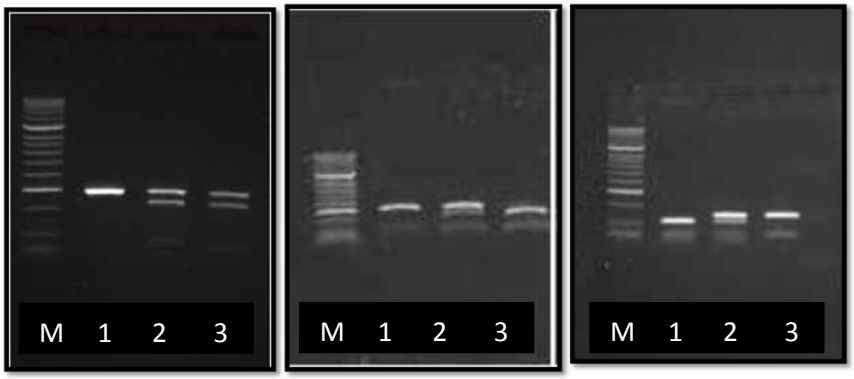
b. Restriction digestion:

Three single-nucleotide polymorphisms (SNPs) in the *IRF6* gene were evaluated: rs2013162, rs2235375, and rs2235371, using restriction digestion of the PCR products, which was carried out using Thermo Scientific**HpyF*3I (*Dde*I), Thermo Scientific *Taq*I, and Thermo Scientific**Mbo*I (*Dpn*II) restriction enzymes (Table 4.4). Positive controls for restriction endonuclease digestion were used for 3 to 5 of the samples in each of the restriction digestion reaction groups. Few study samples (10 cases) were sequenced to insure the quality of the PCR and of restriction digestion as an effective method to identify the presence of SNP variants (see section d, below).

c. Detection of PCR products:

PCR products were detected using agarose gel electrophoresis (2% in 1× TBE buffer), stained with Sybr Safe DNA Gel Stain (Invitrogen, California USA) and visually examined

using a UV transilluminator and photographed using a CCD camera (Gel Doc XR+, Bio-Rad Laboratories, California, USA) (Figure 9 (B) and Figure 9).



(A) (B) (C)

Figure 4.4: Agrose gel electrophoresis of Restriction fragment length polymorphism paterns for IRF6 SNPs. (A) *IRF6* rs2235371; lane M is the 50-bp DNA marker, lane 1 is homozygous C/C and lane 2 & 3 is heterozygous C/T. (B) *IRF6* rs2013162; lane M is the 100-bp DNA marker, lane 1 is homozygous C/C and lane 2 heterozygous C/A and lane 3 is homozygous A/A. (C) *IRF6* rs2235375; lane M is the 100-bp DNA marker, lane 1 is homozygous G/G and lane 2 heterozygous C/G and lane 3 is homozygous C/C (analysis carried out by Edris and AL-Mahdi).

Table 4.4: List of; *IRF6* SNPs mutation sites and alleles; restriction enzymes used and expected fragment size formed for IRF6 gene analysis

Gene	SNP	Position	Allele	Restriction Enzyme	Fragments size after digestion (bp)
IRF6	rs2013162	In 5 exon	**C***/A	*HpyF*3I (TC^TC)	CC 300, AA 220, CA 80
	rs2235375	6,7 splice	**C***/G	*Taq*I (T^CGA)	CC 151, GG 118, CG 33
	rs2235371	In 7 exon	**C***/T	*Mbo*I (^GATC)	CC 232, TT 135, CT 97

*Common allele

d. Sequencing:

Sequencing was performed using the 3500 Genetic Analyzer (Applied Biosystems, California, USA) on a random collection of 10 samples following *IRF6* SNP PCR

amplification to determine the accuracy of the restriction-digestion PCR technique (Figure 4.5). There was no difference between the results from both techniques; therefore, we continued our study using the restriction digestion PCR procedure.

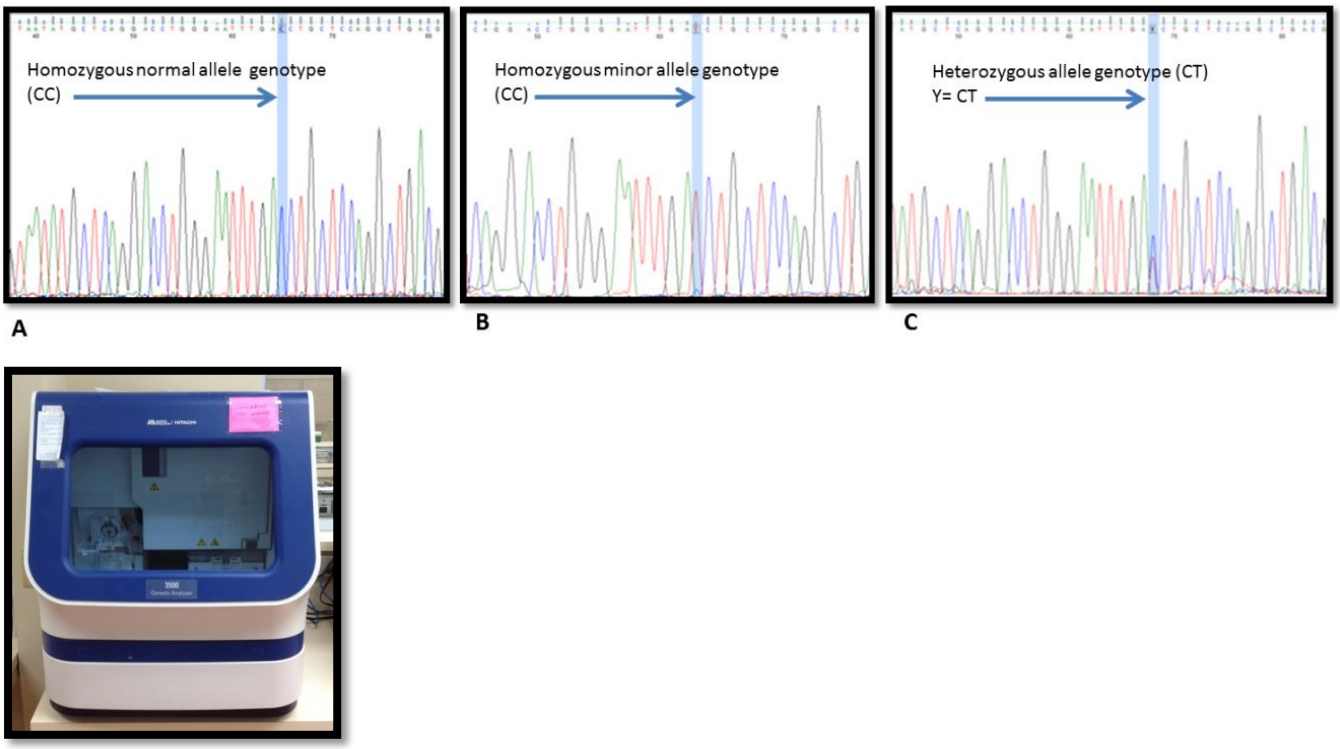


Fig 4.5: The 3500 Genetic Analyzer used for sequencing a random sample of *IRF6* amplification products. A, B and C show the resulting chromatograms from sequencing three samples for rs2235371. (A) A sample containing the homozygous common allele genotype (CC); (B) A sample containing the homozygous rare allele genotype (TT); and (C) A sample that carries the heterozygous allele genotype (CT) (analysis carried out by Edris and Al-Mahdi)

4.2.3.11.3 Genotyping of VAX1:

a. <u>DNA probes:</u>

Two DNA probes were used to detect the *VAX1* rs7078160 and rs4752028 SNPs. They were purchase from Applied Biosystems which was commercial, provided with reference numbers; C_27883342_10 for rs4752028 and C_3197518_10 forrs7078160 (TaqMan® SNP Genotyping Assay).

b. Real-time PCR:

The 7500 Fast Real-Time PCR System (Applied Biosystems), TaqMan® Genotyper Software (Applied Biosystems) and TaqMan® SNP Genotyping Assays were used for genotyping the two *VAX1* SNPs. Once the SNPs were identified within the program, the sample could be applied to the system and the readout was displayed as seen in Figure 4.6. Each colour represents a SNP genotype: red indicates the homozygous minor allele; green represents the heterozygous genotype; and blue represents the homozygous normal allele. Results were analysed using TaqMan® Genotyper Software, a SNP genotyping data analysis tool specifically for use with TaqMan® SNP Genotyping Assays.

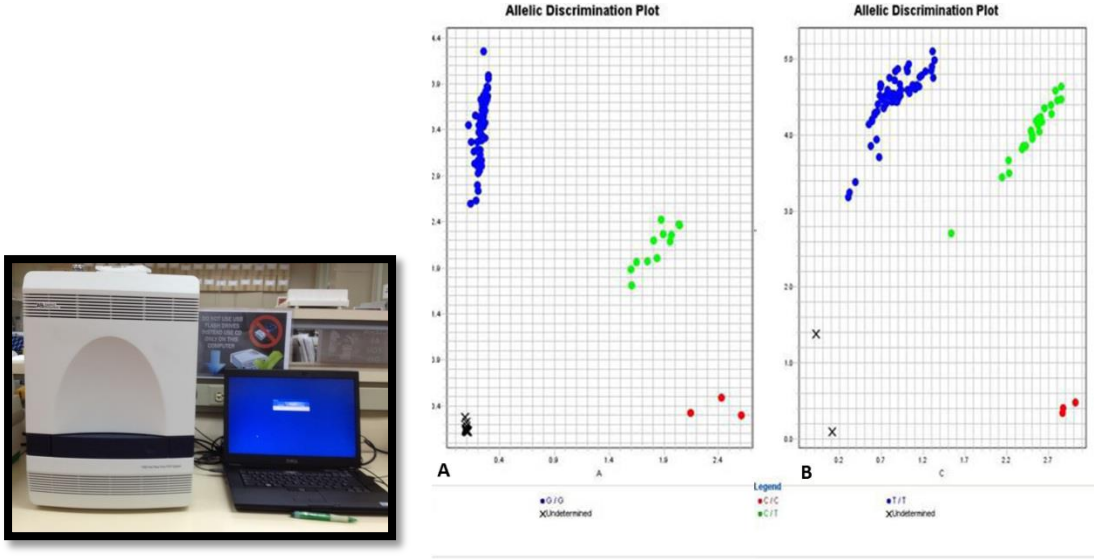


Figure 4.6: Real time PCR used for genotyping *VAX1* rs4752028 and rs7078160 SNPs. A and B are scatter plots (Allelic Discriminations Plots) generated by the 7500 Fast Real Time PCR Software for rs4752028 and rs7078160, respectively.

4.3 Gene-Environment Interaction (GEI)

We hypothesized that environmental factors and maternal exposures strengthen or diminish the effect of maternal rare alleles, which in turn affect the risk of having an infant with NSOFC. These factors include maternal medication and supplementation, maternal diseases,

maternal stress and parental smoking. Oral cleft was not sub-grouped to CL/P and CP, as the number of cases was not large enough to subdivide or stratify. Two study designs were used to measure GEI: case-only and case-control study designs. In the case-only study design, the interaction between maternal SNPs (genotypes and allele) and environmental factors were analysed by measuring the distribution of maternal genotypes and alleles according to exposure/no-exposure to environmental factors among oral cleft cases. In the second approach, the frequencies of maternal genotypes (homozygous common allele, homozygous rare allele and heterozygous allele) in cases were compared to controls according to the different environmental factors.

4.4 Statistical analysis

4.4.1 Questionnaire data entry

The data entry was carried out by the main research coordinator using the SPSS Statistics 19 program (SPSS Inc., Chicago, IL, USA).

4.4.1.1 Double data entry:

Twenty percent of the data points entered into the SPSS program were re-entered to ensure that discrepancies in data entry were minimized.

4.4.2 Descriptive data

The data was analysed and compared using SPSS. Groups were displayed by frequencies and percentages. The type of clefts and their descriptive statistics were displayed by

frequency and percentage values for categorical variables, or by means and standard deviation values for continuous variables. Chi square and t-tests were used for comparisons.

4.4.3 Environmental factors related to oral cleft (CL, CLP and CP)

The different environmental factors and their degree of association to CL, CLP and CP were estimated by measuring the odds ratio (OR), respective 95% confidence intervals (95% CI) and using logistic regression analysis. Chi square and t-tests were used for comparisons.

4.4.4 Genetic factors

Several genetic statistical analyses were carried out for IRF6 (rs2013162, rs2235375, and rs2235371) and VAX1 (rs4752028 and rs7078160) to measure their associations with CL/P and CP.

4.4.4.1 Hardy-Weinberg equilibrium

Hardy-Weinberg equilibrium was determined using an online program (http://www.oege.org/software/hwe-mr-calc.shtml) that compares differences between the observed and expected values of the included homozygous and heterozygous genotype frequencies. The chi-squared goodness of fit test was measured with P-values at 0.05 considered significant.

4.4.4.2 Family-based analysis

Transmission disequilibrium testing (TDT) was performed using the Family-Based Association Test (FBAT) test. PLINK (version v1.07), an open-source whole genome association toolset that is designed to perform a range of basic, large-scale analyses in a

computationally efficient manner, was also used to measure TDT and to separately analyse parental genotype transmission in order to determine the parent of origin (POO) of alleles (Purcell et al., 2007) (http://pngu.mgh.harvard.edu/purcell/plink/).

4.4.4.3 Case-control infant parental triad study design

Infant-parental triad genotypes (homozygous common allele, homozygous rare allele and heterozygous allele) and allele types (rare vs. common) and their degree of association with NSOFC were estimated by measuring the odds ratio (OR) and respective 95% confidence intervals (95% CI). Genotype and allele frequencies among cases (NSOFC, CL/P and CP) and controls were compared using a chi square test with a P-value level of 0.05 using SPSS. Chi square test and the P-value were adjusted using Bonferroni correction methods between cases and controls. The degree of association between genotype and allele frequencies with NSOFC were estimated by measuring the OR and respective 95% confidence intervals (95% CI) using an online program found at http://www.quantpsy.org/chisq/chisq.htm.

4.4.4.4 Haplotype-based association test (HBAT)

The haplotype-based association test (HBAT) was used to investigate haplotypes associated with CL/P and CP.

4.4.4.5 Maternal infant gene-gene interaction

The interaction between maternal variants and infant variants was analysed using general log-linear model using SPSS software. The P-value level indicating a significant interaction prediction was set at a 0.05 confidence interval (CI).

4.4.5 Gene-environmental interaction

Two study designs were used to assess gene-environmental interactions including case-only and case-control.

4.4.5.1 Case-only study design

Interactions between maternal SNPs (genotypes and allele) and environmental factors were analysed by measuring the distribution of maternal genotypes and alleles according to exposure/no-exposure to environmental factors among oral cleft cases. The common allele and null maternal exposure were set as references in our calculation. For the type of drinking water source, tap water with the common allele was set as the reference. Analysis was carried out using chi square test and P-value level at 0.05 using SPSS. Their degrees of association with NSOFC were estimated by measuring the OR and respective 95% confidence intervals (95% CI). If one cell contained a zero value, only the P-value was calculated. No analyses were carried out if more than one cell contained a zero value

Finally, multinominal logistic regression analysis was carried out using SPSS to overcome confounding factors. Factors that showed significant gene-environmental interactions when analysed alone in the previous analysis were entered in the logistic regression.

4.4.5.2 Case-control design

For the case-control design, we compared differences between the frequency of maternal genotypes (homozygous common allele, homozygous rare allele and heterozygous allele) to environmental factors using a chi-square test, and the P-value was adjusted by Bonferroni correction methods between cases and controls using SPSS. OR and 95% CI were measured for factors with P-values≥ 0.05 using MedCalc (user-friendly statistical software). For the type of drinking water source, tap water with the common allele was set as a reference. If

one cell contained a zero value, only the P-value was calculated. No analyses were carried out if more than one cell contained a zero value.

Chapter 5: Results

5.1 Ethical approval

The research and ethical approval took an average of 3 months to be established in most of the medical institutes. However, some institutions took longer time (9 months and more). One hospital, King Faisal Specialized Hospital and research center did not accept the research as they had other similar researches being conducted in their hospital and did not want to distress their patients further. Ethical approvals are presented in appendix A4.

5.2 Sample characteristics

The total number of cases recruited in the two-year study was 217 infant-parental triad; 136 (62.7%) males and 81 (37.3%) females. Of these, 208 (95.9%) answered the questionnaire and were included in the case-control study to measure the effect of environmental factors; 171 (68.8%) infant-parental triad participated in genetic analysis by consenting to saliva sample collection; 133 (61.3%) infant were born in the designated hospitals and their data was used to measure the prevalence of cleft phenotypes. Sample distribution according to recruitment site and NSOFC phenotype description is presented in Appendix A13. Some of the infants (45) included in the prevalence data did not participate in the case-control study.

Two hundred and forty-five normal non-cleft infants participated in the study as controls: 141(57.6) males and 104 (42.4) females. Of these, 244 (99.6%) answered the questionnaire, and 173 (70.6%) complete infant-parental triads participated in the genetic analysis by providing saliva samples. The sample description is displayed in Figure (5.1). However, more infant-mother pairs (189 (74.4%)) agreed to give their saliva.

There was no statistically significant difference between cases and controls with respect to gender.

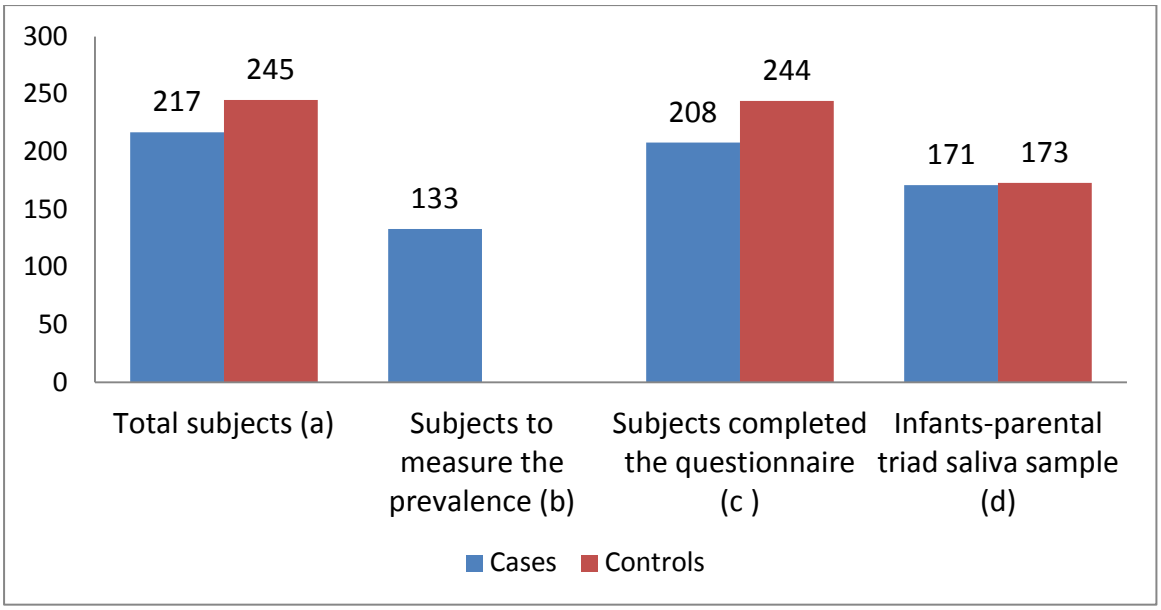


Figure 5.1: Bar chart showing subjects who participated in the study from January 2010 to January 2012. (a) Total subjects recruited for our study. (b) Subjects with NSOFC born in the designated hospitals from January 2010 to January 2012 were compared to the total number of births (114,035 births) to measure the prevalence of NSOFC. (c) Subjects who completed the questionnaire to assess environmental risk factors. (d) Infant-parental triads that agreed to give saliva for genetic analysis

5.3 Part I: Prevalence of NSOFC and the influence of paternal consanguinity among CL/P and CP in Saudi Arabia

From January 2010 to January 2012 there were 114,035 infants born in the designated hospitals. A hundred and thirty-three of these infants were born with NSOFC. Therefore, the total birth prevalence of NSOFC in Saudi Arabia was found to be 1.17/1000 live births.

5.3.1 Characteristics of NSOFC epidemiology in Saudi Arabia

Table 5.1 shows the prevalence of NSOFC cases according to their sub-phenotype and city of origin. The prevalence of CL (0.46/1000 live births) was higher than that of CLP (0.42/1000 live births) and CP (0.28/1000 live births). The prevalence of CL/P was 0.88/1000 live births.

The prevalence of NSOFC was the highest in Maddina (1.88/1000 live births), followed by Riyadh (1.07/1000 live births) with the lowest prevalence in Jeddah (0.8/1000 live births).

Table 5.2 shows the distribution of NSOFC sub-phenotypes in the three cities of Saudi Arabia according to city of birth and gender. In unilateral CL/P, the prevalence of the left-side CL/P sub-phenotype (29 (21.7%) CL cases and 16 (12.5) CLP cases) was higher than for right-side CL/P cases (13 (9.7%) CL or CLP cases). In bilateral CL/P, the frequency of bilateral CLP was 19 (14.2%) cases, which was higher than the frequency of bilateral CL (12 (9%) cases). Complete bilateral CLP (13 (9.7%) cases) frequency was high compared to the frequency of incomplete bilateral CLP (6 (4.5%) cases) and bilateral complete CL (2 (1.5%) cases) with higher male ratio (10 cases (76.9%)) compared to females (3 cases (23.1%)).

The prevalence of NSOFC and CL/P was higher in males (82 cases (61.7%) and 66 cases (65.3%) respectively) than in females (51 cases (38.3%) and 35 cases (34.7%), respectively). On the other hand, females and males showed a similar ratio for CP (1:1). There were statistically significant differences between the three NSOFC phenotypes (CL, CLP and CP) based on gender (P= 0.035). After adjustment using Bonferroni's correction methods, CL was found to occur at levels significantly higher in males than in females. The prevalence of NSOFC was also described and discussed in paper 4, 5& 6 (Appendix B 4, 5 & 6). Bilateral complete CLP was more frequent in males (10 cases) compared to females (three cases)

Table 5.1: Birth prevalence of NSOFC from Jan 2010 to Jan 2011 according to place of birth and OFC phenotype in three large cities in Saudi Arabia.

City	Hospital	Total births	CL	CLP	CP	Total NSOFC	Birth prevalence NSOFC/1000 births
Riyadh							
King Saud Medical City		13,252	6	4	6	16	1.2
Riyadh National Guard Hospital		16,926	2	6	9	17	1.0
King Fahad Medical City		9,827	5	4	1	10	1.0
Total		40,005	13	14	16	43	
Birth prevalence /1000 births			**0.32**	**0.35**	**0.4**		**1.07**
Jeddah							
Al-Messadia Maternity Hospital		13,004	3	3	2	8	0.62
King Abdulaziz University		8,725	3	7	3	13	1.5
National Guard Hospital		9,690	5	4	0	9	0.93
King Fahad Armed Hospital		10,969	3	1	1	5	0.46
Al-Azizia Maternity Hospital		3,508	2	0	0	2	0.57
Total		45,896	16	15	6	37	
Birth prevalence /1000 births			**0.35**	**0.33**	**0.13**		**0.81**
Maddina							
Maddina Maternity Hospital		28134	24	19	10	53	
Births prevalence /1000 births			**0.85**	**0.67**	**0.36**		**1.88**
Three cities together							
Total births in three cities		114,035	53	48	32		133
Total births prevalence in three cities /1000 births			**0.47**	**0.42**	**0.28**		**1.17**

Table 5.2: Distribution of NSOFC sub-phenotypes born in 2010 and 2011 in the three cities of Saudi Arabia according to city of birth and gender.

Phenotype	Sub-phenotype	Riyadh (%)		Jeddah (%)		Maddina (%)		Total (%)
		Male	Female	Male	Female	Male	Female	
CL N= 53 Male: 40 Female: 13	Right incomplete CL	1 (3.7)	1 (6.3)	3 (9.7)	0	3 (9.7)	1 (4.5)	9 (6.7)
	Right complete CL	0	1 (6.3)	0	0	2 (6.5)	1 (4.5)	4 (3)
	Left incomplete CL	6 (22.2)	1 (6.3)	2 (6.5)	2 (8.7)	5 (16.1)	2 (9.1)	18 (14.2)
	Left complete CL	1 (3.7)	1 (6.3)	2 (6.5)	0	6 (19.3)	0	10 (7.5)
	Bilateral incomplete CL	1 (3.7)	0	4 (12.9)	2 (8.7)	3 (9.7)	0	10 (7.5)
	Bilateral complete CL	0	0	1 (3.2)	0	0	1 (4.5)	2 (1.5)
CLP N= 48 Male: 26 Female: 22	Right incomplete CLP	0	0	2 (6.5)	0	0	0	2 (1.5)
	Right complete CLP	1 (3.7)	1 (6.3)	2 (6.5)	2 (8.7)	2 (6.5)	3 (13)	11 (8.2)
	Left incomplete CLP	1 (3.7)	1 (6.3)	0	0	1 (3.2)	1 (4.5)	4 (3)
	Left complete CLP	1 (3.7)	3 (18.8)	0	1 (4.3)	2 (6.5)	5 (21.7)	12 (9)
	Bilateral incomplete CLP	1 (3.7)	0	3 (9.7)	0	0	2 (9.1)	6 (4.5)
	Bilateral complete CLP	4 (14.8)	1 (6.3)	3 (9.7)	2 (8.7)	3 (9.7)	0	13 (9.7)
CP N= 32 Male: 16 Female: 16	CP	10 (37)	6 (37.5)	2 (6.5)	4 (17.3)	4 (12.9)	6 (46.1)	32 (23.9)
	Total	**27 (100)**	**16 (100)**	**24 (100)**	**13 (100)**	**31 (100)**	**22 (100)**	**133 (100)**

5.3.2 Prevalence of parental consanguinity in NSOFC infants

The prevalence of parental consanguinity was measured for NSOFC infants born in the designated hospitals. Paternal consanguinity information was missing for ten cases, which were excluded from this analysis. The number of NSOFC infants with parental consanguinity

was 81 (65.9%) cases: 41 (77.4%) in Maddina; 23 (56.1%) in Riyadh; and 17 (58.6%) in Jeddah, with no significant differences between the values (P= 0.063). Consanguineous marriages involved first cousins in 44 NSOFC cases, which accounted for 54.3% of consanguineous marriages in NSOFC: 22 (53.7%) in Maddina, 12 (52.2%) in Riyadh and 10 (58.8%) in Jeddah.

The prevalence of consanguinity was higher in CP (78.6%) than CL/P (61.1%) but the difference was not significant (P= 0.119). Additionally, the prevalence of severe CL/P (complete clefting of the lip or of bilateral cleft) was higher in infants with consanguineous parents than with non-consanguineous parents, but the differences were not significant (P= 0.123; P= 0.534, respectively). (See Table 5.3).

Table 5.3: Distribution of cases in all three cities combined according to consanguinity and their relationship to NSOFC gender, phenotype and severity.

Type of cleft phenotype	**Consanguinity N (%)**	**Non-consanguinity N (%)**	**Total N (%)**	***X2* (df), P-value OR (95% CI)**
Gender				
Male	49 (63.3)	28 (36.4)	77 (100)	0.45 (1), 0.559
Female	32 (69.6)	14 (30.4)	46 (100)	0.77 (0.35-1.67)
Total	81 (65.9)	42 (34.1)	123* (100)	
Type of NSOFC				
CL/P	59 (61.1)	36 (37.9)	95 (100)	2.61 (1), 0.119
CP	22 (78.6)	6 (21.4)	28 (100)	0.45 (0.17-1.21)
Total	81 (65.9)	42 (34.1)	123* (100)	
Type of CL/P				
CL	31 (63.3)	18 (36.7)	49 (100)	0.06 (1), 0.835
CLP	28 (60.9)	18 (39.1)	33 (100)	1.11 (0.48-2.54)
Total	59 (62.1)	36 (37.9)	95 (100)	
CL extension in CL/P				
Complete CL	33 (70.2)	14 (29.8)	47 (100)	0.39 (1), 0.123
Incomplete CL	24 (54.5)	20 (45.5)	44 (100)	1.96 (0.83-4.65)
Total	57 (62.6)	34 (34.4)	91 (100)	
CL site in CL/P				
Unilateral CL	39 (60)	26 (40)	46 (100)	2.38 (1), 0.534
Bilateral CL	20 (66.7)	10 (33.3)	30 (100)	0.75 (0.3-1.86)
Total	59 (62.1)	36 (37.9)	65 (100)	

*The total number is less than 133 because of ten cases with missing information.

5.4 Part II: Environmental risk factors in the aetiology of NSOFC

Of the 217 NSOFC cases, 208 completed the questionnaire for this part of the study as well as 244 controls. Three of NSOFC group answered the questionnaire but were not examined. Therefore; they were excluded from the analysis**.**

5.4.1 Sample characteristics

Most of the NSOFC cases and controls were of Saudi descent: 184 (88.4%) of the cases and 214 (87.7%) of the controls. Other nationalities included other Middle Eastern countries (16 (7.7%) cases and 15 (6.1%) controls); North African (2 (1%) cases and 5 (2%) controls); Asian (6 (2.9%) cases and 6 (2.5%) controls); and other African countries (4 (1.6%) controls). There was no significant difference between cases and controls with respect to infant nationality (P= 0.32) (see Appendix A14). Prenatal diagnosis was reported in 199 NSOFC cases. Of these, the prevalence of NSOFC cases that were prenatally diagnosed were: 16 (8%) CL, 25 (12.6%) CLP and 4 (2%) CP. There was a statistically significant difference between the three NSOFC sub- phenotypes (P= 0.002).

NSOFC phenotype was described for 205 cases: 78 (38%) CL; 74 (36.8%) CLP; and 53 (25.8%) CP. The cases and controls were aged 18 months or less and were matched in age, gender and hospital where they were ascertained. 71 cases and 104 controls were from Jeddah; Riyadh provided 100 cases and 103 controls; and Maddina provided 34 cases and 37 controls (see Appendix A13). There was no statistically significant difference between cases (129 (62% males and 79 (38%) females) and controls (140 (57.4%) males and 104 (42%) females) with respect to gender (P= 0.3).

The prevalence of NSOFC cases with associated anomalies was 42 (20.8%) cases (9 CL cases (11.7%); 17 CLP cases (23.9%); 16 CP cases (31.4%)). These anomalies were single or combined in NSOFC infants and included cardiovascular defects (17 (27%) cases); limb anomalies (8 (12.7%) cases); renal defects (11 (17.5%) cases); facial defects including eye or ear deformities (18 (28.6%) cases); cranial hydrocephalus defects (4 (6.3%) cases); failure to thrive (2 (3.2%) cases); pulmonary stenosis (2 (3.2) cases); and congenital hernia (1 (1.6%) case). Prenatal diagnosis was more common in CLP (12.6%) than CL (8%) and more common in isolated cases than those combined with associated anomalies (15.6% for isolated CL/P compared to 5% for CL/P with associated anomalies (see table 5.4).

Table 5.4: Case-control comparison according to cleft phenotype and prenatal diagnosis.

Oral cleft type	**Prenatal diagnosis**				
	Yes		No		Total
	Associated anomalies		**Associated anomalies**		
	Yes	No	Yes	No	
CL	3(1.5)	13 (6.5)	6 (3)	55 (27.6)	77[C] (38.7)
CLP	7 (3.5)	18 (9.1)	10 (5.1)	36 (18.1)	71[C] (35.7)
CP	1 (0.5)	3 (1.5)	15 (7.5)	32 (16.1)	51[C] (25.6)
Total	11 (5.5)	34 (17.1)	31 (15.6)	123 (61.8)	199[C] (100)
X^2 (df), **P-value**	0.453 (2), **0.797**		9.34 (2), **0.053**		

[C] Number less than the total cases (Total: 205; CL:78; CLP:74 and CP:53) because of missing information.

5.4.2 Parental age and infant characteristics

The sample was distributed according to parental age and infant characteristics including neonatal weight, neonatal head circumference, neonatal length and number of twin births. The numbers of infants with missing information were 14 cases and 21 controls for infant neonatal weight; 157 cases and 191 controls for neonatal head circumference; 144 cases and

179 controls for neonatal length; and six controls for number of twin births (table 5.5 and 5.6).

The number of infants for which neonatal head circumference and length were able to be recorded were limited: 48 (23.4% of the sample) NSOFC and 53 (21.7% of the total controls) control individuals had data for head circumference; and 61 (29.8% of the sample) NSOFC and 65 (26.6%) of the total controls) control infants had data for infant neonatal length, respectively. There were no significant differences between cases and controls according to parental age and neonatal infant characteristics (P >0.05).

The number of infants that were part of twin births was significantly higher for NSOFC (CL/P phenotype) infants than controls; P= 0.002, OR: 13.2 and 95% CI: 1.69 to 103.4 for NSOFC; P= 0.019, OR: 9.48 and 95% CI: 0.9 to 92.5 for CL; P< 0.001, OR: 24.66 and CI: 3 to 203.94 for CLP) (see Table 5.6).

Table 5.5: Case-control comparison according to parental age in years, and neonatal infant characteristics:

Variable	Group	N	Mean	Significance
Father's age (years)	NSOFC	205	35.89 ± 8.18	0.192
	CL	78	36.75 ± 9.09	0.062
	CLP	73	34.89 ± 6.96	0.982
	CP	53	35.81 ± 8.3	0.457
	Control	**244**	**34.95 ± 6.86**	
Mother's age (years)	NSOFC	202[c]	29.62 ± 5.9	0.484
	CL	78	29.47 ± 5.3	0.777
	CLP	72[c]	29.04 ± 6	0.579
	CP	52[c]	30.65 ± 6.68	0.267
	Control	**244**	**29.24 ± 5.7**	

Infant weight (Kg)	NSOFC	191[c]	2.93 ± 0.58	0.201
	CL	77[c]	2.94 ± 0.54	0.276
	CLP	61[c]	3 ± 0.58	0.113
	CP	53	2.83 ± 0.6	0.732
	Control	**223[c]**	**2.86 ± 0.58**	
Infant neonatal head circumference (cm)	NSOFC	48[c]	34.9 ± 2.2	0.359
	CL	17[c]	34.64 ± 1.98	0.832
	CLP	19[c]	35.34 ± 2.24	0.064
	CP	12[c]	34.92 ± 2.71	0.554
	Control	**53[c]**	**34.52 ± 1.92**	
Infant neonatal length (cm)	NSOFC	61[c]	49.68 ± 4.77	0.937
	CL	22[c]	50.91 ± 4.56	0.23
	CLP	25[c]	48.4 ± 5.5	0.315
	CP	14[c]	50.07 ± 4.03	0.764
	Control	**65[c]**	**49.74 ± 3.7**	

[c] Number less than the total sample (NSOFC: 205; CL:78; CLP:74 and CP:53; controls 244) because of missing information.

Table 5.6: Case-control comparison according to NSOFC sub-phenotype and number of twin or singleton foetuses at birth:

	NSOFC (%)	**CL (%)**	**CLP (%)**	**CP (%)**	**Controls (%)**
Twins	11 (5.4)	3 (3.8)	7 (9.5)	1 (1.9)	1 (0.4)
Singletons	194 (94.6)	75 (96.2)	67 (90.5)	52 (98.1)	237 (99.6)
Total	205 (100)	78 (100)	74 (100)	53 (100)	238[c] (100)
P-value	**0.002****	**0.019****	**<0.001****	**0.24**	
OR (95% CI)	13.2 (1.69–103.4)	9.48 (0.9–92.5)	24.66 (3–203.94)	4.54 (0.28–73.75)	

X^2 =17.99, df=3 and P<0.001**

** Significance value P≤ 0.05

[c] Number less than the total controls (244)

5.4.3 Socioeconomic status (SES)

The SES included information on family income, parental education level and the description of the place of family residence. Table 5.7 shows the distribution of cases and controls according to SES-related proposed risk factors. There were no statistically significant differences between cases (CL, CLP and CP) and controls for family monthly income (X^2= 6.51, df=6, and P= 0.369), paternal education level (X^2= 1.79, df=3, and P= 0.617), and maternal education level (X^2= 2.91, df=3, and P= 0.405). There were more cases living in rural areas compared to controls but the difference was not statistically significant except for CP (P= 0.005, OR: 2.73 and 95% CI: 1.32 to 5.66).

Table 5.7: Case-control comparison according to cleft phenotype and monthly family income in Saudi Riyals (SR), parental education and parental residency if rural or urban.

SES	NSOFC (%)	CL (%)	CLP (%)	CP (%)	Controls (%)
Family income in SR : (X^2 =6.51, df=6, and P= 0.369)					
< 4000	39 (19)	10 (12.8)	19 (26.4)	10 (18.9)	51 (21)
4000-10,000	128 (62.9)	54 (69.2)	39 (53.4)	35 (66)	155 (63.8)
> 10,000	37 (15.3)	14 (17.9)	15 (20.5)	8 (15.1)	37 (15.2)
Total	204[C] (100)	78 (100)	73[C] (100)	53 (100)	243 (100)
P value	**0.683**	**0.22**	**0.23**	**0.95**	
Paternal education level (X^2 =1.79, df=3, and P= 0.617**)**					
< High school	55 (27.7)	17 (21.8)	23 (31.1)	15 (28.8)	68 (28.1)
≥ High school	149 (72.3)	61 (78.2)	51 (68.9)	37 (71.2)	174 (71.9)
Total	204[C] (100)	78 (100)	74 (100)	52[C] (100)	242 (100)
P-value	**0.92**	**0.44**	**0.65**	**0.95**	
OR (95% CI)	0.99 (0.65–1.48)	0.79 (0.44–1.42)	1.14 (0.65–2.01)	1.02 (0.53–1.98)	
Maternal education level (X^2 =2.91, df=3, and P= 0.405)					
< High school	66 (32)	23 (29.5)	21 (28.4)	22 (41.5)	81 (33.5)
≥ High school	139 (68)	55 (70.5)	53 (71.6)	31 (58.5)	161 (66.5)
Total	205 (100)	78 (100)	74 (100)	53 (100)	242 (100)
P-value	**0.666**	**0.514**	**0.46**	**0.27**	
OR (95% CI)	0.92 (0.62–1.36)	0.83 (0.48–1.45)	0.81 (0.46–1.43)	1.4 (0.77–2.59)	
Residency description: (X^2 =8.27, df=3 and P= 0.041**)					
Rural	32 (16.1)	8 (10.3)	11 (14.9)	13 (25)	26 (10.7)
Urban	173 (83.9)	70 (89.7)	63 (85.1)	40 (75)	216 (89.3)
Total	205 (100)	78 (100)	74 (100)	53 (100)	242 (100)

P-value	**0.096**	**0.91**	**0.28**	**0.005****	
OR (95% CI)	1.59 (0.92–2.77)	0.95 (0.47–2.34)	1.5 (0.72–2.68)	2.73 (1.32–5.66)	

** Significance value P≤ 0.05

[C] number less than the total sample (NSOFC: 205; 78; CP:53 and control: 244) because of missing information

5.4.4 Pregnancy planning and the effect of sibling order

Pregnancy planning was reported by more than 40% of case and control families (Table 5.8). Although there was more mothers reporting pregnancy planning in the control (45.4%) compared to CLP (40.5%) and CP (40%) cases, there was no statistically significant difference found between cases and controls with respect to pregnancy planning (CLP: P= 0.504, OR: 0.84, and 95% CI: 0.49 to 1.42; and CP: P= 0.52, OR: 0.81 and 95% CI: 0.44 to 1.52).

Child order was the similar between all groups. The duration between infant and next-oldest sibling pregnancies is presented in Table 5.9. The number of controls with shorter periods between siblings was more than those seen for cases but was statistically significant only for CL. After Bonferroni correction, the number of CL infants with longer duration between infant and next-oldest sibling (three or more years) was higher than for control infants (P= 0.01).

Table 5.8: Case-control comparison to pregnancy planning.

Pregnancy planning	NSOFC (%)	CL (%)	CLP (%)	CP (%)	Controls (%)
Yes	87 (43.3)	37 (48.1)	30 (40.5)	20 (40)	109 (45.4)
No	114 (56.7)	40 (51.9)	44 (59.5)	30 (60)	131 (54.6)
Total	201[C] (100)	77[C] (100)	74 (200)	50[C](100)	240[C] (100)
P-value	**0.681**	**0.713**	**0.504**	**0.52**	
OR (95% CI)	0.92 (0.63–1.35)	1.1 (0.66–1.85)	0.84 (0.49–1.42)	0.81 (0.44–1.52)	

X^2 =1.28, df=3 and P= 0.734

[C] number less than the total sample (NSOFC: 205; CL: 78; CP:53 and control: 244) because of missing information

Table 5.9 Case-control comparison according to infant family order position and the duration between infant and next-oldest sibling.

Variable		NSOFC (%)	CL (%)	CLP (%)	CP (%)	Controls (%)
Child group order X^2 =9.38, df=3 P= 0.67	1st	55 (27.7)	24 (32.9)	18 (25)	13 (24.5)	64 (27.1)
	2nd	40 (20.2)	12 (16.4)	21 (29.2)	7 (13.2)	53 (22.5)
	3rd and 4th	43 (21.7)	17 (23.3)	13 (18.1)	13 (24.5)	42 (17.8)
	5th or more	60 (30.3)	20 (27.4)	20 (27.8)	20 (37.7	77 (32.6)
	Total	193 (100)[C]	73[C]	72[C]	53[C]	236[C]
	P-value	**0.661**	**0.38**	**0.704**	**0.344**	
Duration between the child and their next-oldest sibling in years (y) X^2 =31.51, df=3, P= 0.002**	1 y or less	17 (10.9)	7 (12.7)	7 (12.1)	3 (7)	1 (16.1)
	1.2 - 2 y	44 (28.2)	10 (18.2)	18 (31)	16 (37.2)	58 (32.4)
	2.1- 3 y	33 (21.2)	7 (12.7)	14 (24.1)	12 (27.9)	43 (24.0)
	3.1 y or more	62 (39.7)	31 (56.4)	19 (32.8)	12 (27.9)	67 (37.4)
	Total	**156 (100)[C]**	**55 (100)[C]**	**58 (100)[C]**	**43 (100)[C]**	**169 (100)[C]**
	P-value	0.369	0.01**	0.528	0.721	

** Significance value P≤ 0.05

[C] number less than the total sample (NSOFC: 205; CL: 78; CLP: 74; CP:53 and control: 244) because of missing information

5.4.5. Family history and consanguinity

Factors measured for positive family history included family history for birth defects, family history for NSOFC defects, and parental consanguineous marriages. Family history and parental consanguinity are reported in Tables 5.10 to 5.13.

The prevalence of infants with a family history of birth defect was significantly higher in cases compared to controls, with X^2 =15.89, df=6 and P= 0.014 for the three oral cleft phenotypes, but was significant only for CL (P= 0.012). These congenital anomalies included CL/P (56 NSOFC and 9 controls); CP (10 cases and 6 controls); limb anomalies (1 case and 3 controls); CVD (3 cases and 5 controls); facial deformities (1 case and 2 controls); multiple defects (6 cases and 6 controls); sickle cell anomalies (3 cases and 2 controls); neural tube defects (2 cases and 4 controls); cerebral palsy (5 controls); and other congenital anomalies (5 cases and 35 controls).

There were statistically significant differences between cases and controls with respect to NSOFC family history: NSOFC (P< 0.001, OR: 7.07, 95% CI: 3.88 to 12.88); CL (P= 0.001, OR: 6.51 and 95% CI: 3.18 to 13.32); CLP (P= 0.001, OR: 9.67 and 95% CI: 4.8 to 19.48); and CP (P< 0.001, OR: 4.88 and 95% CI: 2.16 to 11.02).

Information on parental consanguinity was missing from 5 cases and 11 controls. There were more cases reporting parental consanguineous marriages in CP (34 (65.4%) cases) compared to controls (138 (59.2) cases), but the difference was not statistically significant (P>0.05). For the type of consanguineous marriages, 1st cousin consanguineous marriages were greater in cases than controls, especially in CP, but the difference was not statistically significant (P> 0.62).

Table 5.10: Case-control comparison according to family history of birth defects.

Family history	NSOFC (%)	CL (%)	CLP (%)	CP (%)	Controls (%)
Yes	84 (41.2)	32 (41.6)	37 (50)	15 (28.3)	79 (32.4)
No	116 (55.8)	44(57.1)	36 (48.6)	36 (67.9)	161 (66)
unknown	4 (2)	1 (1.3)	1 (1.4)	2 (3.8)	4 (1.6)
Total	204[C] (100)	77[C] (100)	74 (100)	53 (100)	244 (100)
P-value	**0.062**	**0.212**	**0.012****	**0.6**	

X^2 =15.89, df=6 and P= 0.014**

** Significant value P≤ 0.05

[C] number less than the total sample (NSOFC: 205; CL: 78) because of missing information

Table 5.11: Case-control comparison according to family history of NSOFC defects.

Family history	NSOFC (%)	CL (%)	CLP (%)	CP (%)	Controls (%)
Yes	66 (32.2)	23 (29.9)	29 (39.2)	13 (24.5)	15 (6.2)
No	139 (67.8)	54 (70.1)	45 (60.2)	40 (75.5)	228 (93.8)
Total	205 (100)	77[C] (100)	74 (100)	53 (100)	243 (100)
P-value	**<0.001****	**<0.001****	**<0.001****	**<0.001****	
OR (95% CI)	7.07 (3.88–12.88)	6.51 (3.18–13.32)	9.67 (4.8–19.48)	4.88 (2.16–11.02)	

X^2 =63.99 df=3 and P<0.001

** Significance value P≤ 0.05

Table 5.12: Case-control comparison according to parental consanguinity.

Consanguinity	NSOFC (%)	CL (%)	CLP (%)	CP (%)	Controls (%)
Yes	113 (56.5)	39 (50)	40 (57.1)	34 (65.4)	138 (59.2)
No	87 (43.5)	39 (50)	30 (42.9)	18 (34.6)	95 (40.8)
Total	200[C] (100)	78 (100)	70[C] (100)	52[C] (100)	233[C] (100)
P-value	**0.597**	**0.17**	**0.718**	**0.44**	
OR (95% CI)	0.9 (0.62–1.32)	0.7 (0.42–1.17)	0.91 (0.53–1.56)	1.28 (0.68–2.41)	

X^2 =3.47, df=3 and P= 0.325

[C] number less than the total sample (NSOFC: 205; CLP: 74; CP: 53 and control: 244) because of missing information

Table 5.13: Case-control comparison according to type of parental consanguinity among infant with consanguineous parents (113 NSOFC cases; and 129 controls).

Type of consanguinity	NSOFC (%)	CL (%)	CLP (%)	CP (%)	Controls (%)
1st cousins	69 (60.5)	22 (56.4)	25 (62.5)	22 (64.7)	76 (58.9)
1st cousins once removed	8 (7)	4 (10.3)	2 (5)	2 (5.9)	8 (6.2)
2nd cousins	20 (17.5)	7 (17.9)	7 (17.5)	6 (17.6)	18 (14)
Same tribe	16 (13.2)	6 (15.4)	6 (15)	4 (11.8)	27 (20.9)
Total	113(100)	39 (100)	40 (100)	34 (100)	129[C] (100)
P-value	**0.643**	**0.779**	**0.942**	**0.62**	

X^2 =3.46, df=8, and P= 0.903

[C] number less than the total controls with consanguineous parents by nine cases because of missing information

5.4.6 Maternal ingestion of supplements

Maternal ingestion of supplements three months prior to pregnancy and during the 1st trimester period include: multivitamins and/or supplementation with folic acid, calcium, and iron. Tables 5.14 to 5.15 and appendix 15 to 16 show the sample distribution according to maternal supplement ingestion in the three months prior to pregnancy and during the 1st trimester period.

Folic acid supplementation in the pregestation period was more frequently ingested by mothers with CL (14.5%) compared to controls (9.4%), but the difference was not significant (P= 0.22). Folic acid supplementation in the 1st trimester period was significantly more likely to be reported by the mothers of control infants compared to NSOFC mothers (P= 0.009) and CLP mothers (P<0.001). Therefore, can be concluded that folic acid could decreases the risk of having an infant with NSOFC (OR: 0.59 and 95% CI: 0.39 to 0.88) and CLP (OR: 0.34 and 95% CI: 0.2 to 0.58) when ingested in the 1st trimester period.

There were less CL/P mothers using multivitamins supplements compared to controls. However, there was no significant difference (OR: 0.74 and 95% CI: 0.38 to 1.45 for CL; and OR: 0.7 and 95% CI: 0 .3 to 1.4 for CLP)

Calcium supplementation was not ingested in the pre-gestation period by mothers of cases or controls. However, when ingested in the 1st trimester period, it showed a statistically significant decreased risk of having an infant with NSOFC (P= 0.014, OR: 0.37 and 95% CI: 0.16 to 0.84) and with CP phenotype (X^2=5.13 and P= 0.017).

For maternal iron supplementation, no statistically significant differences were identified between cases and controls.

Table 5.14: Case-control comparison according to cleft phenotype and maternal ingestion of folic acid during the three month pre-gestation and 1st trimester periods.

Duration	Folic acid ingestion	NSOFC (%)	CL (%)	CLP (%)	CP (%)	Controls (%)
Pre-gestation X^2 =6.93 df=3 P= 0.074	Yes	17 (8.4)	11 (14.5)	3 (4.1)	3 (5.7)	23(9.4)
	No	186 (91.6)	65 (85.5)	71 (95.9)	50 (94.3)	221 (90.6)
	Total	203[C] (100)	76[C] (100)	74 (100)	53 (100)	244 (100)
	P-value	**0.79**	**0.22**	**0.16**	**0.41**	
	OR (95% CI)	0.915 (0.48–1.75)	1.62 (0.75–3.5)	0.42 (0.12–1.45)	0.6 (0.17–2.07)	
1st trimester X^2 =18.03 df=3 P<0.001	Yes	127 (62.6)	55 (72.4)	37 (50)	35 (66)	181 (74.2)
	No	76 (37.4)	21 (27.6)	37 (50)	18 (34)	63 (25.8)
	Total	203[C] (100)	76 [C] (100)	74 (100)	53 (100)	244 (100)
	P-value	**0.009****	**0.71**	**<0.001****	**0.18**	
	OR (95% CI)	0.59 (0.39–0.88)	0.9 (0.5–1.6)	0.34 (0.2–0.58)	0.65 (0.34–1.22)	

** Significance value P≤ 0.05

[C] number less than the total sample (NSOFC: 205; CL: 78) because of missing information

Table 5.15: Case-control comparison according to maternal ingestion of calcium supplements in the 1st trimester.

Calcium supplement	**NSOFC (%)**	**CL (%)**	**CLP (%)**	**CP (%)**	**Controls (%)**
Yes	8 (3.9)	5 (6.6)	3 (4.1)	0	24 (9.8)
No	197 (96.1)	73 (93.4)	71 (95.9)	53 (100)	220 (90.2)
Total	205 (100)	78 (100)	74 (100)	53 (100)	244 (100)
P-value	**0.014****	**0.347**	**0.125**	**0.024****	
OR (95% CI)	0.37 (0.16–0.84)	0.62 (0.23–1.7)	0.38 (0.11–1.31)	a	

X^2=8.05, df=3 and P= 0.045**

** Significance value P≤ 0.05

The OR and CI were not measured because one group contains zero value.

5.4.7 Maternal disease

Diseases experienced and maternal exposure to X-rays by NSOFC mothers and controls were recorded for the three months prior to pregnancy and 1st trimester periods and are illustrated in Tables 5.16 to 5.20 and appendix (A17 to A21). These diseases include: common cold/ flu, renal diseases, asthma, convulsions, diabetes, high blood pressure, vaginal bleeding, and fever. Furthermore, maternal exposure to X-rays and the reason for exposure was recorded for the same periods of time.

Maternal illness in the three month pre-gestation period was reported significantly more often by NSOFC mothers and by mothers of infants with all three sub-phenotypes compared to mothers of controls. For NSOFC: P< 0.001, OR: 2.6 and 95% CI: 1.64 to 4.17; for CL, P= 0.024, OR: 2.04 and 95% CI: 1.1 to 3.82; for CLP, P< 0.001, OR: 3.62 and 95% CI: 2 to 6.53; and for CP, P= 0.013, OR: 2.39, 95% CI: 1.19 to 4.81. However, for illness during the first trimester period, there was a statistically significant difference between cases and controls for NSOFC (P= 0.017, OR: 1.6 and 95% CI: 1.09 to 2.35) and CL (P= 0.037, OR: 1.74 and 95% CI: 1.04 to 2.94).

Table 5.17 shows the distribution of NSOFC mothers reporting a common cold/ flu experience during the three months prior to pregnancy and the 1st trimester period. Maternal cold/flu infection in the three months prior to pregnancy was significantly reported more frequently by mothers of case infants compared to mothers of control infants for all three NSOFC sub-phenotypes (NSOFC, P< 0.001, OR: 3.99 and 95% CI: 2.14 to 7.43; CL, P= 0.014, OR: 2.45, 95% CI: 1.23 to 6.28; CLP, P< 0.001, OR: 6.34 and 95% CI: 3.11 to 13.18; and CP, P= 0.031, OR: 2.66 and 95% CI: 1.06 to 6.63). For the 1st trimester period, there was a statistically significant difference between the total CP cases compared to controls in

maternal reporting of common cold/ flu infection (P= 0.037, OR: 2.03 and 95% CI: 1.03 to 3.98). However, this difference was not statistically significant for CL/P.

Maternal fever was also significantly associated with NSOFC, with more NSOFC and CLP mothers reporting having experienced fever in the three month pre-gestation period than control mothers (P= 0.048, OR: 1.98 and 95% CI: 1.01 to 3.63 for NSOFC and P= 0.008, OR: 2.78 and 95% CI: 1.28 to 6.05 for CLP) (Table 5. 18).

Other diseases including renal diseases, asthma, convulsions, diabetes, high blood pressure, and vaginal bleeding were rarely reported as having been experienced by mothers three months during and prior to pregnancy (See appendix A17 to A21). In addition, these disorders were not associated with NSOFC, with the exception of diabetes, which was significantly associated with CP if experienced in the 1st trimester period (P= 0.018, OR: 4.8 and 95% CI: 1.16 to 19.84) (see Table 5.19).

For maternal exposure to X-rays during the 1st trimester period of pregnancy, four (1.9%) NSOFC cases reported exposure to X-rays compared to 6 (2.5%) of controls with no statistically significant difference (P= 0.705). For maternal exposure to X-rays in the 1st trimester period, four (5.1%) CLP cases reported exposure to X-rays compared to three (1.2%) controls with statistically significant difference (P= 0.05, OR: 4.59, and 95% CI: 1-21). The general reason given for X-ray exposure were dental X-ray procedures (see Table 5.20).

Table 5.16: Case-control comparison according to maternal illness during the three month pre-gestation and 1st trimester periods.

Duration	Illness	NSOFC (%)	CL (%)	CLP (%)	CP (%)	Controls (%)
Pre-gestation X^2 =21.36 df= 3 P<0.001	Yes	63 (30.6)	20 (25)	28 (37.8)	15 (28.3)	35 (14.4)
	No	142 (69.4)	58 (75)	46 (62.2)	38 (71.7)	208 (85.6)
	Total	205 (100)	78 (100)	74 (100)	53 (100)	243[C] (100)
	P-value	**<0.001****	**0.024****	**<0.001****	**0.013****	
	OR (95% CI)	2.6 (1.64–4.17)	2.04 (1.1–3.82)	3.62 (2–6.53)	2.39 (1.19–4.81)	
1st trimester X^2 =6.65 df= 3 P= 0.084	Yes	88 (42.7)	35 (43.4)	30 (40.5)	23 (43.4)	77 (31.8)
	No	117 (57.3)	43 (56.6)	44 (59.5)	30 (56.6)	165 (68.2)
	Total	205[C] (100)	78 (100)	74 (100)	53 (100)	242[C] (100)
	P-value	**0.017****	**0.037****	**0.167**	**0.094**	
	OR (95% CI)	1.6 (1.09–2.35)	1.74 (1.04–2.94)	1.46 (0.86–2.5)	1.7 (0.95–3.1)	

** Significance value
[C] number less than the total sample (Control: 244) because of missing information

Table 5.17: Case-control comparison according to maternal self-report of experiencing common-cold/ flu during the three month pre-gestation and 1st trimester periods.

Duration	Cold/flu	NSOFC (%)	CL (%)	CLP (%)	CP (%)	Controls (%)
Pre-gestation	Yes	42 (20.9)	12 (15.8)	22 (29.7)	8 (15.1)	15 (6.2)
X^2 =28.76	No	163 (79.1)	66 (84.2)	52 (70.3)	45 (84.9)	227 (93.8)
df= 3	Total	205 (100)	78 (100)	74 (100)	53 (100)	242[C] (100)
P<0000	**P-value**	**<0.001****	**0.014****	**<0.001****	**0.031****	
	OR (95% CI)	3.99 (2.14–7.43)	2.45 (1.23–6.28)	6.4 (3.11–13.18)	2.66 (1.06–6.63)	
1st trimester	Yes	50 (24.8)	16 (21.1)	18 (24.3)	16 (30.2)	42 (17.4)
X^2 =4.92	No	155 (74.9)	62 (78.9)	56 (75.7)	37 (69.8)	200 (82.6)
df= 3	Total	205 (100)	78 (100)	74 (100)	53 (100)	242[C] (100)
P= 0.178	**P-value**	**0.054**	**0.53**	**0.183**	**0.037****	
	OR (95% CI)	1.57 (0.99–2.48)	1.23 (0.65–2.34)	1.53 (0.81–2.86)	2.03 (1.03–3.98)	

** Significance value P≤ 0.05

[C] number less than the total sample (control: 244) because of missing information

Table 5.18: Case-control comparison according to maternal fever during the three month pre-gestation and 1st trimester periods.

Duration	Fever	NSOFC (%)	CL (%)	CLP (%)	CP (%)	Controls (%)
Pre-gestation	Yes	26 (12.7)	8 (10.5)	13 (17.6)	5 (9.4)	17 (7)
X^2 =7.1	No	179 (87.3)	70 (89.5)	61 (82.4)	48 (91.2)	225 (93)
df= 3	Total	205 (100)	78 (100)	74 (100)	53 (100)	242[C] (100)
P= 0.069	**P-value**	**0.048****	**0. 36**	**0.008****	**0.562**	
	OR (95% CI)	1.98 (1.01–3.63)	1.51 (0.63–3.65)	2.78 (1.28–6.05)	1.3 (0.48–3.87)	
1st trimester	Yes	34 (16.6)	17 (22.4)	9 (12.2)	8 (15.1)	36 (14.9)
X^2 =2.97	No	171 (83.4)	61 (77.6)	65 (78.8)	45 (84.9)	206 (85.1)
df= 3	Total	205 (100)	78 (100)	74 (100)	53 (100)	242[C] (100)
P= 0.397	**P-value**	**0.636**	**0. 155**	**0.534**	**0.967**	
	OR (95% CI)	1.13 (0.68–1.9)	1.59 (0.84–3.04)	0.78 (0.36–1.71)	1.02 (0.44–2.33)	

** Significance value P≤ 0.05

[C] number less than the total sample (control: 244) because of missing information

Table 5.19: Case-control comparison according to maternal diabetes during three month pre-gestation and 1st trimester periods.

Duration	Diabetes	NSOFC (%)	CL (%)	CLP (%)	CP (%)	Controls (%)
Pre-gestation X^2 =4.6 df= 3 P= 0.204	Yes	3 (1.5)	0	2 (2.7)	1 (1.9)	1 (0.4)
	No	202 (98.5)	78 (100)	72 (97.3)	52 (98.1)	241 (99.6)
	Total	205 (100)	78 (100)	74 (100)	53 (100)	242[C] (100)
	P-value	**0.242**	**0.988**	**0.123**	**0.241**	
	OR (95% CI)	3.56 (0.37–34.5)	a	6.69 (0.59–74.974)	4.58 (0.28–74.37)	
1st trimester X^2 =5.55 df= 3 P= 0.136	Yes	9 (4.4)	3 (3.9)	2 (2.7)	4 (7.5)	4 (1.7)
	No	196 (95.6)	75 (96.1)	72 (97.3)	49 (92.5)	238 (98.3)
	Total	205 (100)	78 (100)	74 (100)	53 (100)	242[C] (100)
	P-value	**0.088**	**0.26**	**0.573**	**0.018****	
	OR (95% CI)	2.7 (0.8–8.9)	2.38 (0.52–10.87)	1.63 (0.29–9.09)	4.8 (1.16–19.84)	

** Significance value P≤ 0.05

a. The OR and CI were not measured because one group contains zero value.

b. [C] Number less than the total number (controls: 244) because of missing information

Table 5.20: Case-control comparison according to maternal exposure to X-rays in the three months pre-gestation and 1st trimester periods.

Duration	X-ray	NSOFC (%)	CL (%)	CLP (%)	CP (%)	Controls (%)
Pre-gestation X^2 =2.67 df= 3 P= 0.445	Yes	4 (1.9)	1 (1.3)	3 (3.8)	0	6 (2.5)
	No	201 (98.1)	77 (98.7)	71 (96.2)	53 (100)	238 (97.5)
	Total	205 (100)	78 (100)	74 (100)	53 (100)	244 (100)
	P-value	**0.705**	**0.542**	**0.473**	**0.468**	
	OR (95% CI)	0.78 (0.22–2.81)	0.51 (0.06–4.34)	1.68 (0.41-6.87)	a	
1st trimester X^2 =4.44 df= 3 P= 0.218	Yes	7 (3.4)	2 (2.6)	4 (5.1)	1 (1.9)	3 (1.2)
	No	198 (96.6)	76 (97.4)	70 (94.9)	52 (98.1)	241 (98.8)
	Total	205 (100)	78 (100)	74 (100)	53 (100)	244 (100)
	P-value	**0.122**	**0.417**	**0.05****	**0.709**	
	OR (95% CI)	2.81 (0.72–11.01)	2.11 (0.35–12.89)	4.59 (1–21)	1.54 (0.16–15.15)	

** Significance value P≤ 0.05

a. The OR and CI were not measured because one group contains zero value.

5.4.8 Maternal medications ingestion

Medications ingested by NSOFC and control mothers during the three months prior to pregnancy and 1st trimester period were recorded. These medications included antibiotics, antipyretics, contraceptives, anti-emetics, and progesterone (Tables 5.28 to 5.32).

Table 5.21 shows the sample distribution according to maternal antibiotics ingestion in the three month pre-gestation and the 1st trimester periods. There was a statistically significant difference between NSOFC, including CL/P, and controls in relationship to maternal antibiotics, in the three month pre-gestation period (P= 0.003, OR: 2.81 and 95% CI: 1.38 to

5.72 for NSOFC; P= 0.012, OR: 2.96 and 95% CI: 1.22 to 7.16 for CL; and P= 0.03, OR: 2.64 and 95% CI: 1.07 to 6.55 for CLP). For the 1st trimester period, there was a statistically significant difference between NSOFC, including CL, and controls (P= 0.023, OR: 1.95 and 95% CI: 1.09 to 3.51 for NSOFC and P= 0.003, OR: 2.82 and 95% CI: 1.39 to 5.74 for CL). Although there was more frequent CP mothers using antibiotics in the pregestation and 1st trimester periods compared to controls, the differences were not significant (P>0.05).

Table 5.22 shows the sample distribution according to maternal antipyretic in the three month pre-gestation and the 1st trimester periods. Maternal ingestion of antipyretic medications in the 1st trimester period showed a statistically significant difference between NSOFC and controls (P= 0.033, OR: 0.48 and 95% CI: 0.24 to 0.94). However, this difference was not significant for NSOFC sub-phenotypes.

For anti-emetic medication, Table 5.23 shows a significant difference between NSOFC and CL, and controls in relation to maternal ingestion of anti-emetics in the first trimester period (NSOFC: P= 0.042, OR: 1.97 and 95% CI: 1.02 to 3.8; CL: P= 0.019, OR: 2.34 and 95% CI: 1.06 to 5.75). However, this difference was not significant for the various CLP and CP sub-phenotypes.

Contraceptive ingestion showed no statistically significant differences between cases and controls in the pre-gestation and 1st trimester periods (Appendix 22). Although more mothers in the control group reported using progesterone tablet compared to NSOFC and its three phenotypes, the differences were not significant (P>0.05) (see Appendix A23).

Other medications that were rarely reported by mothers were: anti-hypertensive, anti-coagulant, anti-fungal, insulin, motilium (domperidone; dopamine antagonist anti-sickness medicine), thyroxin, anti-convulsive, and anti-histamine. ingestion of these medications was reported by only one to three mothers. Cortisone ingestion was reported by 8 mothers: 6

(2.9%) cases and 2 (0.8%) controls, and there was no statistically significant difference between cases and controls (P= 0.096, OR: 3.6 and 95% CI: 0.72 to 18.05).

Table 5.21: Case-control comparison according to cleft phenotype and maternal ingestion of antibiotics in the three month pre-gestation and 1st trimester periods.

Duration	**Anti-biotics**	**NSOFC (%)**	**CL (%)**	**CLP (%)**	**CP (%)**	**Controls (%)**
Pre-gestation X^2 =7.79 df= 3 P= 0.051	Yes	25 (12.3)	10 (13.3)	9 (12.2)	6 (11.3)	12 (4.9)
	No	177 (87.7)	65 (86.7)	65 (87.8)	47 (88.7)	232 (95.1)
	Total	202[C] (100)	75[C] (100)	74 (100)	53 (100)	244 (100)
	P-value	**0.003****	**0.012****	**0.030****	**0.08**	
	OR (95% CI)	2.81 (1.38–5.72)	2.96 (1.22–7.16)	2.64 (1.07–6.55)	2.44 (0.87–6.82)	
1st trimester X^2 =7.95 df=3 P= 0.047**	Yes	32 (15.5)	16 (21.1)	9 (12.3)	7 (13.2)	21 (8.6)
	No	170 (84.5)	60 (78.9)	64 (77.7)	46 (86.8)	223 (91.4)
	Total	202[C] (100)	76[C] (100)	73[C] (100)	53 (100)	244 (100)
	P-value	**0.023****	**0.003 ****	**0.35**	**0.31**	
	OR (95% CI)	1.95 (1.09–3.51)	2.82 (1.39–5.74)	1.48 (0.65–3.39)	1.59 (0.64–3.97)	

** Significance value P≤ 0.05

[C] Number less than the total number (NSOFC: 205 cases, CL: 78 cases and controls: 244) because of missing information.

Table 5.22: Case-control comparison according to maternal antipyretic medication ingestion during the three month pre-gestation and the in 1st trimester periods.

Duration	**Anti-pyretic**	**NSOFC (%)**	**CL (%)**	**CLP (%)**	**CP (%)**	**Controls (%)**
Pre-gestation X^2 =4.26 df=3 P= 0.235	Yes	14 (6.8)	6 (6.2)	6 (8.1)	2 (3.8)	30 (12.3)
	No	191 (93.2)	72 (93.3)	68 (91.9)	51 (96.2)	214 (87.7)
	Total	205 (100)	78(100)	74 (100)	53 (100)	244 (100)
	P-value	**0.033****	**0.266**	**0.323**	**0.088**	
	OR (95% CI)	0.48 (0.24–0.94)	0.59 (0.24–1.49)	0.62 (0.25–1.58)	0.28 (0.06–1.21)	
1st trimester X^2 =3.77 df=3 P= 0.287	Yes	25 (12.3)	9 (11.2)	8 (10.8)	8 (15.4)	45 (18.8)
	No	179 (87.7)	69 (88.8)	66 (89.2)	44 (84.6)	195 (81.2)
	Total	204[c] (100)	78 (100)	74 (100)	52[c] (100)	240 (100)
	P-value	**0.056**	**0.121**	**0.12**	**0.6**	
	OR (95% CI)	0.6 (0.35–1.02)	0.55 (0.26–1.18)	0.5 (0.24–1.19)	0.8 (0.35–1.82)	

** Significance value P≤ 0.05

[C] Number less than the total number (NSOFC: 205 cases, CL: 78 cases and controls: 244) because of missing information.

Table 5.23: Case-control comparison according to maternal exposure to anti-emetic medications during the three month pre-gestation and 1st trimester periods.

Duration	Anti-emetic	NSOFC (%)	CL (%)	CLP (%)	CP (%)	Controls (%)
Pre-gestation	Yes	1 (0.5)	0	1 (1.4)	0	0
X^2 =5.04	No	204 (99.5)	78 (100)	73 (98.6)	53 (100)	244 (100)
df=3	Total	205 (100)	78 (100)	74 (100)	53 (100)	244 (100)
P= 0.169	**P-value**	**0.437**	**a**	**0.07**	**a**	
	OR (95% CI)	2.10 (0.14-88)	a	4.36 (3.56–5.34)	a	
1st trimester	Yes	25 (12.1)	12 (14.5)	7 (9.6)	7 (13.2)	16 (6.6)
X^2 =7.16	No	181 (87.9)	66 (85.5)	67(90.4)	46 (86.8)	228 (93.4)
df=3	Total	205 (100)	78 (100)	74 (100)	53 (100)	244 (100)
P= 0.067	**P-value**	**0.043****	**0.019****	**0.401**	**0.108**	
	OR (95% CI)	1.97 (1.02–3.8)	2.34 (1.06–5.75)	1.49 (0.59–3.77)	2.17 (0.84–5.57)	

** Significance value P≤ 0.05

a. Cannot be analysed because it contain zero values

5.4.9 Maternal stress

Maternal stressors during the three month period prior to pregnancy and during the 1st trimester were measured by recording information on: maternal reporting of having experienced stress; maternal reporting of having experienced family problems; maternal depression; maternal reporting having experienced severe morning sickness, threatened abortion and/or abdominal pain (Tables 5.24 to 5.26 and Appendix 24 to 26).

There was a significant difference between cases and controls regarding mothers reporting having experienced stress and having family problems during the three month pre-gestation and/or the 1st trimester periods. Stress and family problems were significantly related to NSOFC and its sub-phenotypes and experience of these increased the chances of having an infant with NSOFC (maternal stress: P< 0.001, OR: 2.1 and 95% CI: 1.43 to 3.11 for NSOFC; P= 0.04, OR: 1.74 and 95% CI: 1.01 to 3 for CL; P= 0.001, OR: 2.53 and 95% CI: 1.48 to 4.32 for CLP; and P= 0.009, OR: 2.23 and 95% CI: 1.2 to 4.09 for CP; and maternal family problems: P= 0.001, OR: 1.97 and 95% CI: 1.31 to 2.95 for NSOFC; P= 0.04, OR: 1.79 and 95% CI: 1.03 to 3.13 for CL; P= 0.004, OR: 2.21 and 95% CI: 1.28 to 3.84 for CLP; and P= 0.04, OR: 1.96 and 95% CI: 1.04 to 3.67 for CP). In addition, maternal depression in the pre-gestation and 1st trimester periods was reported more in NSOFC mothers compared to controls and showed a statistically significant relationship with CP when it occurred in the pre-gestation period (P= 0.049, OR: 3.87 and 95% CI: 1.003 to 14.93) and with CL when it occurred in the 1st trimester (P= 0.032, OR: 2.96 and 95% CI: 1.1 to 7.96)

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Appendix A26 shows the sample distribution according to maternal experience of abdominal pain in the three months prior to pregnancy and the 1st trimester period. Abdominal pain was reported significantly more in NSOFC cases and CP compared to controls in the 1st trimester period (P= 0.04, OR: 2.06 and 95% CI: 1.03 to 4.11 for NSOFC and P= 0.004, OR: 3.59 and 95% CI: 1.43 to 8.83 for CP). Other factors that increase maternal stress such as threatened abortion and severe morning sickness were not statistically related to NSOFC (P> 0.05).

Table 5.24: Case-control comparison according to cleft phenotype and maternal experience of stress in the pre-gestation and/or 1st trimester periods.

Stress	**NSOFC**	**CL (%)**	**CLP (%)**	**CP (%)**	**Controls (%)**
Yes	95 (46.6)	32 (41.6)	38 (51.4)	25 (47.2)	71 (29.1)
No	109 (53.4)	45 (58.4)	36 (58.6)	28 (52.8)	173 (90.9)
Total	204[C] (100)	77[C] (100)	74 (100)	53 (100)	244 (100)
P-value	**<0.001****	**0.04****	**0.001****	**0.009****	
OR (95% CI)	2.1 (1.43–3.11)	1.74 (1.01–3)	2.53 (1.48–4.32)	2.23 (1.2–4.09)	

X^2=16.84, df=3 and P= 0.001**
** Significance value P≤ 0.05
[C] number less than the total sample (NSOFC: 205; and CL:78) because of missing information

Table 5.25: Case-control comparison according to cleft phenotype and maternal experience of family problems during the three month pre-gestation and/or 1st trimester periods.

Family problem	**NSOFC (%)**	**CL (%)**	**CLP(%)**	**CP (%)**	**Controls (%)**
Yes	78 (38.4)	28 (36.4)	30 (41.1)	20 (37.7)	59 (24.2)
No	125 (61.6)	49 (63.6)	43 (58.9)	33 (62.3)	185 (75.8)
Total	203[C] (100)	77[C] (100)	73[C] (100)	53 (100)	244 (100)
P-value	**0.001****	**0.04****	**0.004****	**0.04****	
OR (95% CI)	1.97 (1.31–2.95)	1.79 (1.03–3.13)	2.21 (1.28–3.84)	1.96 (1.04–3.67)	

X^2 =12.41, df=3 and P= 0.006**

** Significance value P≤ 0.05

[C] number less than the total sample (NSOFC: 205; and CL:78) because of missing information

Table 5.26: Case-control comparison according to NSOFC phenotype and maternal depression in the three months pre-gestation and 1st trimester periods.

Duration	Depression	NSOFC (%)	CL (%)	CLP (%)	CP (%)	Controls (%)
Pre-gestation X^2 =4.52 df=3 P= 0.211	Yes	9 (4.4)	2 (2.6)	3 (4.1)	4 (7.5)	5 (2.1)
	No	196 (95.6)	76 (97.4)	71 (95.9)	49 (92.5)	237 (97.9)
	Total	205 (100)	78 (100)	74 (100)	53 (100)	242 (100)
	P-value	**0.163**	**0.794**	**0.34**	**0.049****	
	OR (95% CI)	2.17 (0.71–6.57)	1.25 (0.24–6.56)	2.01 (0.47–8.64)	3.87 (1.003–14.93)	
1st trimester X^2 =6.03 df=3 P= 0.11	Yes	17 (8.3)	8 (10.2)	5 (6.8)	4 (7.5)	9 (3.7)
	No	188(91.7)	70 (89.8)	69 (93.2)	49 (92.5)	233 (96.3)
	Total	205 (100)	78 (100)	74 (100)	53 (100)	242 (100)
	P-value	**0.045****	**0.032****	**0.273**	**0.228**	
	OR (95% CI)	2.34 (1.02–5.37)	2.96 (1.1–7.96)	1.87 (0.61–5.78)	2.11 (0.63–7.14)	

** Significance value P≤ 0.05

5.4.10 Parental smoking

Parental smoking, and the type and intensity of smoking were recorded for the three months prior to pregnancy and the 1st trimester period. Table 5.27 shows the sample distribution according to cleft phenotype and maternal smoking in the three month pre-gestation and 1st trimester periods. Only six (2.9%) mothers reported smoking in the pre-gestation period and three (1.5%) mothers reported smoking in the 1st trimester period. Therefore, the differences between cases and controls were not significant (P= 0.35 and P= 0.47, respectively). Maternal type and intensity of smoking differences were too insignificant to report.

Paternal smoking and their type and intensity in the three month pre-gestation and 1st trimester periods are reported in Tables 5.28 to 5.32. There were more CP fathers reporting smoking in general and smoking tobacco in both periods compared to controls. However, the differences were not significant (P= 0.098). On the other hand, more fathers in the control group reported smoking compared to CL fathers (in the pregestation period: P= 0.018, OR: 0.51, and 95% CI: 0.28 to 0.92, for smoking in general; and P= 0.001, OR: 0.29 and 95% CI: 0.13 to 0.63, for tobacco smoking; and in the 1st trimester period: P= 0.002, OR: 0.28 and 95% CI: 0.13 to 0.62, for smoking in general; and P= 0.001, OR: 0.3 and 95% CI: 0.14 to 0.65, for tobacco smoking). Intense parental smoking (more than 20 cigarettes per day) was significantly associated with an increased risk of having an infant with NSOFC (P= 0.013, OR: 3.07 and 95% CI: 1.25 to 7.55) and CP (P= 0.012, OR: 5.23, 95 % CI 1.33 to 20.58).

A statistically significant relationship was found between paternal waterpipe smoking and NSOFC including CL/P. For NSOFC: in the pre-gestation period: P= 0.013, OR: 2.33 and 95% CI: 1.18 to 4.62; and in the 1st trimester period P= 0.016, OR: 2.47 and 95% CI: 1.16 to 5.27. For CL: in the pre-gestation period P= 0.04, OR: 2.45 and 95% CI: 1.04 to 5.77; and in

the 1st trimester period P= 0.046, OR: 2.56 and 95% CI: 1 to 6.62. For CLP: in the pre-gestation period P< 0.001, OR: 3.83 and 95% CI: 1.73 to 8.48; and in the 1st trimester period P= 0.001, OR: 4.11 and 95% CI: 1.73 to 9.77. However, the relationship was not significant for CP (P> 0.05).

Paternal waterpipe smoking was sub-grouped into Jorak and Moasel. Jorak smoking was associated with a statistically significant increased risk of having an infant with NSOFC including CL/P if it occurred in the three months prior to pregnancy: P= 0.001, OR: 6.34 and 95% CI: 1.8 to 22.23 for NSOFC; P= 0.01, OR: 5.58 and 95% CI 1.3 to 23.95 for CL; and P< 0.001, OR: 9.62 and 95% CI: 2.48 to 37.27 for CLP. For the first trimester period, paternal Jorak smoking was also associated with significantly increased NSOFC risk; P= 0.01, OR: 4.66 and 95% CI: 1.28 to 16.93 for NSOFC; P= 0.03, OR: 4.55 and 95% CI: 1 to 2.82 for CL; P= 0.002, OR: 7.1 and 95% CI: 1.73 to 29.16 for CLP. However, although NSOFC fathers smoked Moasel more frequently (15 (7.3%)) and CL/P (6 (8%) for CL and 8 (10.8%) for CLP) compared to controls (12 (4.9%)), the relationship was not statically significant: P= 0.287, OR: 1.53 and 95% CI: 0.7 to 3.34 for NSOFC; P= 0.338, OR: 1.64 and 95% CI: 0.59 to 4.52 for CL; and P= 0.072, OR: 2.31 and 95% CI: 0.91 to 6 for CLP. For CP the relationship was also not significant (P= 0.321, OR: 0.37 CI: 0.05 to 2.89). The results of the two types of waterpipe smoking (Moasel and Jorak) are presented in Appendix A27.1 and A27.2.

Maternal second-hand smoking was reported by 45 (22%) of NSOFC mothers and 21 (28.4%) CLP mothers compared to 47 (19.3%) of controls. As mothers were mainly house wives (87.3% of NSOFC and 86.4% of controls' mothers), family members are the primary source for maternal second hand smoking.

Although maternal second-hand smoking was more frequently reported in mothers of NSOFC and its sub-phenotypes compared to controls, and especially among those with who were exposed more than 12hours/ week (25% NSOFC, 40% CL and 23% CP compared to 19% controls), there were no statistically significant differences between cases and controls (P> 0.05) (see Tables 5.33 and 5.34). For tobacco smoking fathers, 77.8% of CL mothers reported exposure to 2nd hand smoking compared to 46.1% of controls but the difference was not significant (OR: 4.1 and 95% CI: 0.8 to 21.03). However, there were significantly more CLP and CP mothers reported being exposed to 2nd hand smoking compared to controls (OR: 10.54 and 95% CI: 2.29 to 48.64 for CLP; and OR: 15.23 and 95% CI: 1.9 to 122.3 for CP) among tobacco smoking fathers. Comparison between cases and controls according to cleft phenotype and maternal exposure to paternal tobacco smoking is presented in appendix (A27.3).

Table 5.27: Case-control comparison according to cleft phenotype and maternal smoking in the three month pre-gestation and 1st trimester periods.

Duration	Smoking	NSOFC (%)	CL (%)	CLP (%)	CP (%)	Controls (%)
pre-gestation	Yes	6 (2.9)	2 (2.7)	4 (4.1)	0	10 (4.1)
X^2 =3.03	No	198 (97.1)	75 (97.3)	70 (95.9)	53 (100)	234 (95.9)
df= 3	Total	204[C] (100)	77[C] (100)	74 (100)	53 (100)	244 (100)
P= 0.387	P-value	0.35	0.67	0.99	0.15	
	OR (95% CI)	0.6 (0.2–1.7)	0.71 (0.15–3.37)	0.99 (0.27–3.71)	a	
1st trimester	Yes	3 (1.5)	2 (2.6)	1 (1.4)	0	6 (2.5)
X^2 =1.66	No	201 (98.5)	75 (97.4)	73 (98.6)	53 (100)	236 (97.5)
df= 3	Total	204[C] (100)	77[C] (100)	74 (100)	53 (100)	242 (100)
P= 0.647	P-value	0.47	0.92	0.57	0.25	
	OR (95% CI)	0.6 (0.15–2.4)	1.08 (0.21–5.48)	0.55 (0.07–4.61	1.23 (1.16–1.3)	

[C] number less than the total sample (NSOFC: 205; and CL: 78) because of missing information.

a. The OR and CI were not measured because one group contains zero value.

Table 5.28: Case-control comparison according to cleft phenotype and paternal smoking during the three month pre-gestation and/or 1st trimester periods.

Duration	Smoking	NSOFC (%)	CL (%)	CLP (%)	CP (%)	Controls (%)
Pregestation X^2=17.63 df= 3 P= 0.001**	Yes	74 (36.3)	17 (22.1)	33 (44.6)	24 (45.3)	90 (36.9)
	No	130 (63.7)	60 (77.9)	41 (55.4)	29 (54.7)	154 (63.1)
	Total	204[C] (100)	77[C] (100)	74 (100)	53 (100)	244 (100)
	P-value	0.967	0.018**	0.236	0.258	
	OR (95% CI)	1.01 (0.69–1.48)	0.51 (0.28–0.92)	1.38 (0.81–2.33)	1.14 (0.78–2.58)	
1st trimester X^2=17.02 df= 3 P= 0.001**	Yes	52 (25.5)	8 (10.4)	22 (29.7)	22 (41.5)	70 (29)
	No	152 (74.5)	69 (89.6)	52 (70.3)	31 (58.5)	171 (71)
	Total	204[C] (100)	77[C] (100)	74 (100)	53 (100)	241[C] (100)
	P-value	0.452	0.002**	0.91	0.079	
	OR (95% CI)	0.85 (0.56-1.29)	0.28 (0.13-0.62)	1.03 (0.58-1.83)	1.73 (0.94-3.2)	

X^2 =10.77, df= 2, P= 0.013**

** Significance value P≤ 0.05

[C] number less than the total sample (NSOFC: 205; CL: 78; and controls: 244) because of missing information.

Table 5.29: Case-control comparison according to cleft phenotype and paternal smoking of tobacco in the three month pre-gestation and 1st trimester periods.

Duration	Tobacco	NSOFC (%)	CL (%)	CLP (%)	CP (%)	Controls (%)
Pregestation X^2=17.63 df= 3 P= 0.001**	Yes	57 (27.9)	9 (11.7)	25 (33.8)	23 (43.4)	77 (31.6)
	No	147 (72.1)	68 (88.3)	49 (66.2)	30 (56.6)	167 (68.4)
	Total	204[C] (100)	77[C] (100)	74 (100)	53 (100)	244 (100)
	P-value	0.524	0.001**	0.717	0.098	
	OR (95% CI)	0.88 (0.58–1.32)	0.29 (0.13–0.63)	1.11 (0.64–1.93)	1.66 (0.91–3.06)	
1st trimester X^2=17.03 df= 3 P= 0.001	Yes	51 (25.4)	8 (10.7)	21 (28.8)	22 (41.5)	71 (29.1)
	No	150 (74.6)	67 (89.3)	52 (71.2)	31 (58.5)	173 (70.9)
	Total	201[C] (100)	75[C] (100)	73[C] (100)	53 (100)	244 (100)
	P-value	0.38	0.001**	0.93	0.08	
	OR (95% CI)	0.83 (0.54–1.26)	0.3 (0.14–0.65)	0.97 (0.55–1.73)	1.7 (0.94–3.2)	

** Significance value P≤ 0.05

[C] number less than the total sample (NSOFC: 205; and CL: 78) because of missing information.

Table 5.30: Case-control comparison according to cleft phenotype and paternal smoking of ≥ 20 cigarettes/ day in the pregestation period and/or 1st trimester.

Smoking ≥ 20 cigarettes/ day	**NSOFC (%)**	**CL (%)**	**CLP (%)**	**CP (%)**	**Controls (%)**
Yes	30 (73.8)	4 (71.4)	11 (61.1)	15 (83.3)	22 (47.8)
No	11 (26.2)	1 (28.6)	7 (38.9)	3 (16.7)	24 (52.2)
Total	41[C] (100)	5[C] (100)	18[C] (100)	18[C] (100)	46 [C] (100)
P-value	0.013**	0.187	0.380	0.012**	
OR (95% CI)	3.07 (1.25–7.55)	4.18 (0.43–40.39)	1.64 (0.54–5)	5.23 (1.33–20.58)	

X^2 =7.26, df=3 and P= 0.064

** Significance value P≤ 0.05

[C] number less than the total fathers that smoked tobacco (NSOFC: 57; CL: 9; CLP: 25; CP: 23; and controls: 77) because of missing information.

Table 5.31: Case-control comparison according to cleft phenotype and paternal non-smoking tobacco in the three month pre-gestation and 1st trimester periods.

Duration	Nonsmoking Tobacco	NSOFC (%)	CL (%)	CLP (%)	CP (%)	Controls (%)
Pre-gestation X^2=7.13 df=9 P= 0.672	Yes	2 (1)	1 (1.3)	1 (1.4)	0	1`
	No	201 (99)	76 (98.7)	72 (98.6)	53 (100)	243
	Total	203[C] (100)	77[C] (100)	73[C] (100)	53 (100)	244
	P-value	0.477	0.38	0.36	0.64	
	OR (95% CI)	2.4 (0.22 - 27.1)	3.27 (0.2 - 52.93)	3.34 (0.21 - 54.19)	a	
1st trimester X^2=3.84 df= 3 P= 0.28	Yes	3 (1.5)	1 (1.3)	1 (1.4)	1 (2)	0
	No	199 (98.5)	76 (98.7)	73 (98.6)	50 (98)	244 (100)
	Total	202[C] (100)	77[C] (100)	74 (100)	51[C] (100)	244 (100)
	P-value	0.057	0.07	0.068	0.029**	
	OR (95% CI)	a	a	a	a	

** Significance value P≤ 0.05

[C] number less than the total sample (NSOFC: 205; CL: 78; and CLP: 74) because of missing information

a. The OR and CI were not measured because one group contains zero value

Table 5.32: Case-control comparison according to cleft phenotype and paternal waterpipe smoking in the three month pre-gestation and 1st trimester periods.

Duration	Paternal waterpipe	NSOFC (%)	CL (%)	CLP (%)	CP (%)	Controls (%)
Pre-gestation	Yes	26 (12.2)	10 (13)	14 (18.9)	2 (3.8)	14 (5.7)
	No	178 (87.8)	67 (87)	60 (81.1)	51 (96.2)	230 (94.3)
X^2=15.17	Total	204[C] (100)	77[C] (100)	74 (100)	53 (100)	244 (100)
df= 3	P-value	0.013**	0.04**	<0.001**	0.58	
P= 0.002**	OR (95% CI)	2.33 (1.18–4.62)	2.45 (1.04–5.77)	3.83 (1.73–8.48)	0.639 (0.14–2.9)	
1st trimester	Yes	21 (10.5)	8 (10.5)	12 (16.2)	1 (1.9)	11 (4.5)
	No	182 (89.5)	68 (98.5)	62 (83.8)	52 (98.1)	232 (95.5)
X^2=15.28	Total	203[C] (100)	76[C] (100)	74 (100)	53 (100)	243 (100)
df= 3	P-value	0.016**	0.046**	0.001**	0.372	
P= 0.002**	OR (95% CI)	2.47 (1.16–5.27)	2.56 (1–6.62)	4.11 (1.73–9.77)	0.4 (0.05–3.18)	

** Significance value P≤ 0.05

[C] number less than the total sample (NSOFC: 205; and CL: 78) because of missing information

Table 5.33: Case-control comparison according to cleft phenotype and maternal second-hand smoking in the three month pre-gestation and 1st trimester periods.

Maternal 2nd-hand smoking	NSOFC (%)	CL (%)	CLP (%)	CP (%)	Controls (%)
Yes	45 (22)	10 (13)	21 (28.4)	14 (26.4)	47 (19.3)
No	159 (78)	67 (87)	53 (71.6)	39 (73.6)	197 (80.7)
Total	204[C] (100)	77[C] (100)	74 (100)	53 (100)	244 (100)
P-value	0.482	0.237	0.088	0.231	
OR (95% CI)	1.18 (0.75–1.87)	0.64 (0.31–1.34)	1.68 (0.93–3.06)	1.52 (0.76–3.03)	

X^2 =6.93, df=3 and P= 0.074
[C] number less than the total sample (NSOFC: 205; CL: 78; and CLP: 74) because of missing information

Table 5.34: Case-control comparison according to duration of maternal secondary smoke exposure in hours per week among the different cleft phenotype.

Number of hours/week (h/w)	NSOFC (%)	CL (%)	CLP (%)	CP (%)	Control (%)
<6 h/w (reference)	11 (25)	4 (40)	4 (19)	3 (23.1)	8 (19)
6-12 h/w	18 (30.9)	3 (30)	11 (52.4)	4 (30.8)	22 (52.4)
P value **OR (95% CI)**	0.357 1.68 (0.56-5.07)	0.13 3.66 (0.67-20.1)	1 1 (0.25-4.06)	0.404 2.06 (0.38-11.31)	
>12 h/w	15 (34.1)	3 (30)	6 (28.6)	6 (46.2)	12 (28.6)
P value **OR (95% CI)**	0.875 1.1 (0.34-3.6)	0.436 2 (0.35-11.44)	1 1 (0.21-4.7)	0.733 0.75 (0.14-3.9)	
Total	44[C] (100)	10 (110)	21 (100)	13[C] (100)	42[C] (100)

X^2 =4.3, df=6 and P= 0.637
[C] number less than the total number of maternal secondary smoke exposure (NSOFC: 205; CP: 14; and controls: 47) because of missing information

5.4.11 Domestic exposure

Domestic maternal exposure during the three month period prior to pregnancy and the 1st trimester includes exposure to any chemicals, solvents (thinner and/or acetone), pesticides, and incense. Table 5.35 shows the sample distribution according to maternal exposure to chemicals during the three month pre-gestation and the 1st trimester periods. There was a significant difference between CLP cases and controls in maternal reporting of exposure to chemicals in the pre-gestation period (P= 0.003, OR: 2.23 and 95% CI: 1.3 to 3.82) and in the 1st trimester period (P= 0.043, OR: 1.74 and 95% CI: 1.02 to 2.99). Table 6.36 shows that there was also a significant difference between CLP cases and controls in the proportion of mothers reporting exposure to solvents (P= 0.011, OR: 2.25 and 95% CI: 1.18 to 4.28) in the pre-gestation period.

Maternal exposure to incense in the first trimester showed significant association with a reduced risk of having an infant with NSOFC (P= 0.037, OR: 0.67 and 95% CI: 0.46 to 0.98) and CL (P= 0.005, OR: 0.47 and 95% CI: 0.28 to 0.8). Incense exposure in the pre-gestation period was also associated with a reduced risk of having an infant with CL (P= 0.011, OR: 0.51 and 95% CI: 0.3 to 0.86) (Table 5.37).

Other domestic maternal exposures such as pesticides (Appendix A28), microwaves and computers were not associated with NSOFC and were reported in appendix A29.

Table 5.35: Case-control comparison according to cleft phenotype and maternal exposure to any chemicals reported by the mothers in the three month pre-gestation and the 1st trimester periods.

Duration	Chemicals	NSOFC (%)	CL (%)	CLP (%)	CP (%)	Controls (%)
Pre-gestation	Yes	68 (33)	17 (22.4)	34 (45.9)	17 (32.1)	67 (27.7)
	No	136 (67)	60 (77.6)	40 (54.1)	36 (67.9)	175 (72.3)
X^2=11.95 df=3	Total	204[C] (100)	77[C] (100)	74 (100)	53 (100)	242 (100)
P= 0.008	**P-value**	**0.208**	**0.366**	**0.003****	**0.515**	
	OR (95% CI)	1.3 (0.87 - 1.94)	0.76 (0.41 - 1.39)	2.23 (1.3 - 3.82)	1.24 (0.65 - 2.35)	
1st trimester	Yes	69 (33.8)	20 (25)	31 (41.9)	18 (34)	72 (29.8)
	No	135 (66.2)	57 (75)	43 (58.1)	35 (66)	170 (70.2)
Chi-squared =5.49	Total	204[C] (100)	77[C] (100)	74 (100)	53 (100)	242 (100)
df=3	**P-value**	**0.437**	**0.469**	**0.043****	**0.502**	
P= 0.139	OR (95% CI)	1.17 (0.79 - 1.75)	0.81 (0.45 - 1.45)	1.74 (1.02 - 2.99)	1.2 (0.66 - 2.34)	

** Significance value P≤ 0.05

[C] number less than the total sample (NSOFC: 205; and CL: 78) because of missing information.

Table 5.36: Case-control comparison according to cleft phenotype and maternal exposure to solvents (thinner and/or acetone) in the three month pre-gestation and the 1st trimester periods.

Duration	Solvent	NSOFC (%)	CL (%)	CLP (%)	CP (%)	Controls (%)
Pre-gestation	Yes	33(16.2)	8 (10.4)	19 (25.7)	6 (11.3)	33 (13.5)
X^2=9.17	No	171 (83.8)	69 (89.6)	55 (74.3)	47 (88.7)	211 (86.5)
df= 3	Total	204[C] (100)	77[C] (100)	74 (100)	53 (100)	244 (100)
P= 0.027	**P-value**	**0.43**	**0.473**	**0.011****	**0.701**	
	OR (95% CI)	1.23 (0.37 - 082)	0.74 (0.33 - 1.7)	2.25 (1.18 - 4.28)	0.83 (0.33 - 2.11)	
1st trimester	Yes	28 (13.7)	10 (12)	12 (16.2)	6 (11.3)	31 (12.7)
X^2=0.871	No	176 (86.3)	67 (88)	62 (83.8)	47 (88.7)	213 (87.3)
df= 3	Total	204[C] (100)	77[C] (100)	74 (100)	53 (100)	244 (100)
P= 0.832	**P-value**	**0.883**	**0.86**	**0.404**	**0.821**	
	OR (95% CI)	1.04 (0.6 - 1.8)	0.93 (0.42 - 2.06)	1.36 (0.66 - 2.81)	0.9 (0.35 - 2.28)	

** Significance value P≤ 0.05

[C] number less than the total sample (NSOFC: 205; and CL: 78) because of missing information

Table 5.37: Case-control comparison according to cleft phenotype and maternal exposure to incense during the pre-gestation and the first trimester periods.

Duration	Incense	NSOFC (%)	CL (%)	CLP	CP (%)	Controls (%)
Pre-gestation	Yes	88 (43.1)	28 (36.8)	35 (47.3)	25 (47.2)	129 (52.9)
X^2=7.05	No	116 (56.9)	49 (63.2)	39 (52.7)	28 (52.8)	115 (47.1)
df= 3	Total	204[C] (100)	77[C] (100)	74 (100)	53 (100)	244 (100)
P= 0.07	**P-value**	**0.058**	**0.011****	**0.348**	**0.401**	
	OR (95% CI)	0.7 (0.48 - 1.01)	0.51 (0.3 - 0.86)	0.78 (0.46 - 1.31)	0.78 (0.43 - 1.41)	
1st trimester	Yes	87 (42.6)	27 (35.5)	34 (45.9)	26 (49.1)	130 (53.3)
X^2=8.61	No	117 (57.4)	50 (64.5)	40 (54.1)	27 (50.9)	114 (46.7)
df= 3	Total	204[C] (100)	77[C] (100)	74 (100)	53 (100)	244 (100)
P= 0.035	**P-value**	**0.037****	**0.005****	**0.228**	**0.519**	
	OR (95% CI)	0.67 (0.46 - 0.98)	0.47 (0.28 - 0.8)	0.73 (0.43 - 1.22)	0.82 (0.45 - 1.49)	

** Significance value P≤ 0.05

[C] number less than the total sample (NSOFC: 205; and CL: 78) because of missing information

5.4.12 Type of main maternal drinking water

The main maternal drinking water sources in Saudi Arabia include tap, bottled, Zamzam and well waters. Table 5.38 shows the sample distribution according to type of maternal drinking water. The prevalence of mothers drinking bottled water was greater than other water sources: 131 (64.9%) of cases; and 159 (66%) of controls. There were statistically significant differences between NSOFC and CL/P compared to controls with respect to the type of maternal drinking water source (P= 0.001 for NSOFC; P= 0.016 for CL; P= 0.02 for CLP and P= 0.225 for CP). After adjusting for pairwise comparisons between cases and controls for the four water source types using Bonferroni correction methods, there were significantly more NSOFC and CL/P mothers who had drunk well water compared to controls. In addition, there were significantly more control mothers who had drunk Zamzam water compared to NSOFC and CL/P mothers (P<0.05).

Table 5.38: Case-control comparison according to type of maternal drinking water.

Type of water	NSOFC (%)	CL (%)	CLP (%)	CP (%)	Controls (%)
Tap	56 (28.2)	19 (24.7)	21 (29.6)	16 (30.2)	60 (24.9)
Bottled	131 (64.9)	54 (70.1)	43 (60.6)	34 (64.2)	159 (66)
Well	11 (5.4)	4 (5.2)	5 (7)	2 (3.8)	3 (1.2)
Zamzam	3 (1.5)	0	2 (2.8)	1 (1.9)	19 (7.9)
Total	201[C] (100)	77[C] (100)	71[C] (100)	53 (100)	241[C] (100)
P-value	**0.001****	**0.016****	**0.02****	**0.225**	

X^2=18, df=9, and P= 0.033**

[C] number less than the total sample (NSOFC: 205; and CL: 78;CLP: 74 ; and controls:244) because of missing information

** Significance value P< 0.05

5.4.13 Multiple logistic regression analysis

Table 5.39 shows variables that had statistical significant relationship with NSOFC, CLP and CP in multiple logistic regression analyses with the significant adjusted OR and 95% CI.

Stepwise logistic regression analysis identified eight factors as significant predictors for NSOFC, five as significant predictors for CL, seven as significant predictors for CLP and four as significant predictors for CP risk. For NSOFC the factors were: family history for NSOFC (P<0.001, OR: 8.78 and 95% CI: 4.43 to 17.39); maternal common cold/flu during the three months pre-gestation (P= 0.002, OR: 3.28 and 95% CI: 1.57 to 6.64); maternal ingestion of anti-emetic medication during the 1st trimester (P= 0.006, OR: 3.00 and CI: 1.38 to 6.55); antipyretic medication (P= 0.004, OR: 0.27 and 95% CI: 0.11 to 0.66); Calcium supplementation in the 1st trimester (P= 0.053, OR: 0.4 and 95% CI: 0.15 to 1.01); mother complaining of being under stress during pregestation and 1st trimester period (P= 0.003, OR: 2 and 95% CI: 1.27 to 3.14); paternal waterpipe smoking (P= 0.03 and OR: 2.38 and 95% CI: 1.09 to 5.22); and maternal drinking water (P= 0.007). Maternal Zamzam drinking water shows a marginally significant effect compared to bottled water (P= 0.033, OR: 0.23 and 95% CI: 0.06 to 0.89), Tap water (P= 0.049, OR: 0.25 and 95% CI: 0.06 to 0.99) and well water (P= 0.001, OR: 0.03 and 95% CI: 0.005 to 0.23).

For CL, the predictor variables were: family history for NSOFC (P<0.001, OR: 8.79 and 95% CI: 3.92 to 19.68); antibiotics during the pregestation (P= 0.033, OR: 3.01 and CI: 1.1 to 8.61) and during the 1st trimester (P= 0.005, OR: 3.07 and 95% CI: 1.4 to 6.73); mothers complaining of family problems (P= 0.011, OR: 2.29 and 95% CI: 1.21 to 4.35); and incense exposure in the 1st trimester (P= 0.031, OR: 0.51 and 95% CI: 0.27 to 0.94).

For CLP the associated variables were: family history for NSOFC (P< 0.001, OR: 14.73 and 95% CI: 5.99 to 36.17); common cold/flu during the pre-gestation period (P< 0.001, OR:

5.82 and 95% CI: 2.38 to 14.25); folic acid in the 1st trimester (P= 0.007, OR: 0.09 and 95% CI: 0.14 to 0.51) maternal stress (P= 0.002, OR: 3 and 95% CI: 1.49 to 6.03); paternal waterpipe smoking (P= 0.001, OR: 5.74 and 95% CI: 2.07 to 15.9); maternal exposure to chemicals in the pregestation period (P= 0.003, OR: 2.91 and 95% CI: 1.44 to 5.89) and maternal main water source (P= 0.001). Maternal Zamzam drinking water shows a significant effect compared to well water (P= 0.001, OR: 0.02 and 95% CI: 0.002 to 0.2),

For CP the factors were: family history (P< 0.001. OR: 5.89 and 95% CI: 2.36 to 14.74); maternal common cold/flu in the pregestation period (P= 0.029, OR: 2.28 and 95% CI: 1.09 to 4.77); abdominal pain in the 1st trimester (P= 0.001, OR: 5.81 and 95% CI: 2.05 to 16.45); and maternal stress (P= 0.028, OR: 2.1 and 95% CI: 1.08 to 4.06).

Table 5.39: Multiple logistic regression analysis showing the most significant factors related to NSOFC, CL, CLP and CP.

Factors	**P-value** **OR (95% Cl)**			
	NSOFC*	CL**	CLP***	CP****
Factors increasing the chance of NSOFC				
Family history	<0.001** 8.78 (4.43-17.39)	<0.001** 8.79 (3.92-19.68)	<0.001** 14.7 (5.99-36.2)	<0.001** 5.89(2.36-14.74)
Common cold/flu pre-gestation	0.002** 3.28 (1.57-6.64)	a	<0.001** 5.82 (2.38-4.25)	0.029 2.28 (1.09-4.77)
Abdominal pain in the 1st trimester	a	a	a	0.001 5.81 (2.05-16.45)
Antibiotic in pre-gestation	a	0.033 3.01 (1.1-8.61)	a	a

Factors	P-value OR (95% Cl)			
	NSOFC*	CL**	CLP***	CP****
Antibiotic in 1st trimester	a	0.005 3.07 (1.4-6.73)	a	a
Anti-emetic medication	0.0063 (1.38-6.55)	a	a	a
Maternal stress	0.003 2 (1.27-3.14)	a	0.002 3 (1.49-6.03)	0.028 2.1 (1.08-4.06)
Family problems	a	0.011 2.29 (1.21-4.35)	a	a
Paternal waterpipe smoking	0.03 2.38 (1.09-5.22)	a	0.001 5.74 (2.07-15.9)	a
Maternal exposure to chemical in the pregestation period	a	a	0.003 2.91 (1.44–5.89)	a
Factors decreasing the chance of NSOFC:				
Folic acid in the 1st trimester	a	a	0.007 0.09 (0.14-0.51)	a
Calcium supplementation	0.053 0.4 (0.15-1.01)	a	a	a
Antipyretic in pregestation	0.004 0.27 (0.11-0.66)	a	a	a
Incense in the 1st trimester	a	0.031 0.51 (0.27-0.94)	a	a
Maternal main water source	**0.007**	**a**	**0.001**	**a**
Zamzam water compared to bottled water	0.033 0.23 (0.06-0.89)	a	0.67 0.71 (0.14-3.48)	a
When compared to	0.049	a	0.701	a

Factors	P-value OR (95% Cl)			
	NSOFC*	CL**	CLP***	CP****
tap water	0.25 (0.06-0. 99)		0.72 (0.14-38)	
When compared to well water	0.001 0.03 (0.005-0.23)	a	0.001 0.02 (0.002-0.2)	a

* Factors entered in the multiple regression included: family history of NSOFC, twins versus singleton birth frequency , antibiotics in the pre-gestation and 1st trimester periods, antipyretic medication in the pre-gestation period, anti-emetic medication in the 1st trimester, maternal illness in the pre-gestation and 1st trimester periods, flu/ common cold during pre-gestation and 1st trimester periods, fever in the pre-gestation period, folic acid in the 1st trimester periods, calcium supplementation in the 1st trimester period, mothers complaining of stress, mothers with family problems, abdominal pain in the 1st trimester period, paternal waterpipe smoking, and type of maternal drinking water source.

** Factors entered in the multiple regression include: family history of NSOFC, twins versus singleton birth frequency, antibiotics in the pre-gestation and 1st trimester periods, anti-emetic medication, maternal illness in the pre-gestation period, flu/ common cold in the pre-gestation period, mothers complaining of stress, mothers with family problems, paternal waterpipe smoking, type of maternal drinking water source, and maternal incense exposure in the pre-gestation and 1st trimester periods.

***Factors entered in the multiple regression includes: family history of NSOFC, twins versus singleton birth frequency, folic acid in the 1st trimester period, antibiotic in the pre-gestation period, maternal illness in the pre-gestation period, flu/ common cold in the pre-gestation period, maternal fever in the pre-gestation period, mothers complaining of stress, mothers with family problems, paternal waterpipe smoking, maternal exposure to chemicals in the pre-gestation and 1st trimester periods, maternal solvent exposure during the pre-gestation period, and type of maternal drinking water source.

**** Factors entered in the multiple regression include: family history, calcium supplementation in the 1st trimester period, maternal illness in pre-gestation period, flu/ common cold in the pre-gestation and 1st trimester period, abdominal pain in the 1st trimester period, mothers complaining of stress, and mothers with family problems.

a. No values either because the variable were not entered in the logistic regression or had no significant relationship with cleft.

(Appendix A30 shows variables not in the equation which is not significant)

5.5 Genetic analysis results

Genetic analysis was carried out for *IRF6* (rs2013162, rs2235375, and rs2235371) and *VAX1* (rs4752028 and rs7078160) and their associations with CL/P and CP using 171 case parental triad family-based approach and case-control infant-parental triad-approach by comparing cases to 189 infant-parental triad controls. However, 16 fathers in the control group did not provide saliva. In addition, 10 of the cases were not sub-grouped according to cleft phenotype, resulting in 127 CL/P and 34 CP cases in our analysis.

5.5.1 Hardy-Weinberg equilibrium

Table 5.40 shows the Hardy-Weinberg equilibrium calculations for the *IRF6* (rs2013162, rs2235375 and rs2235371) and *VAX1* (rs4752028 and rs7078160) genotypes in case and control infant-parental triads. *VAX1* rs4752028 showed no significant differences between the observed and expected values for the included homozygous and heterozygous genotype frequencies with P-values greater than 0.05, indicating that the case and control triad samples were in Hardy-Weinberg equilibrium at this locus. However, the other loci were not in Hardy-Weinberg equilibrium, including; among NSOFC cases: *IRF6* rs2013162 paternal gene variance (χ^2 = 7.85 and P = 0.002) and maternal *IRF6* rs2235375 gene variance (χ^2 = 6.04 and P = 0.05); among controls: *IRF6* rs2013162 maternal gene variance (χ^2 = 8.92 and P = 0.01) and *VAX1* rs7078160 paternal gene variance (χ^2 = 21.76 and P< 0.001); among both cases and controls: *VAX1* rs7078160 maternal gene variance (χ^2 = 18.23 and P< 0.001, and χ^2 = 20.9 and P< 0.001) respectively.

Table 5.40 The observed frequency (OF) and expected frequency (EF) for the included genotypes (*IRF6* and *VAX1*) using the Hardy Weinberg frequency calculation.

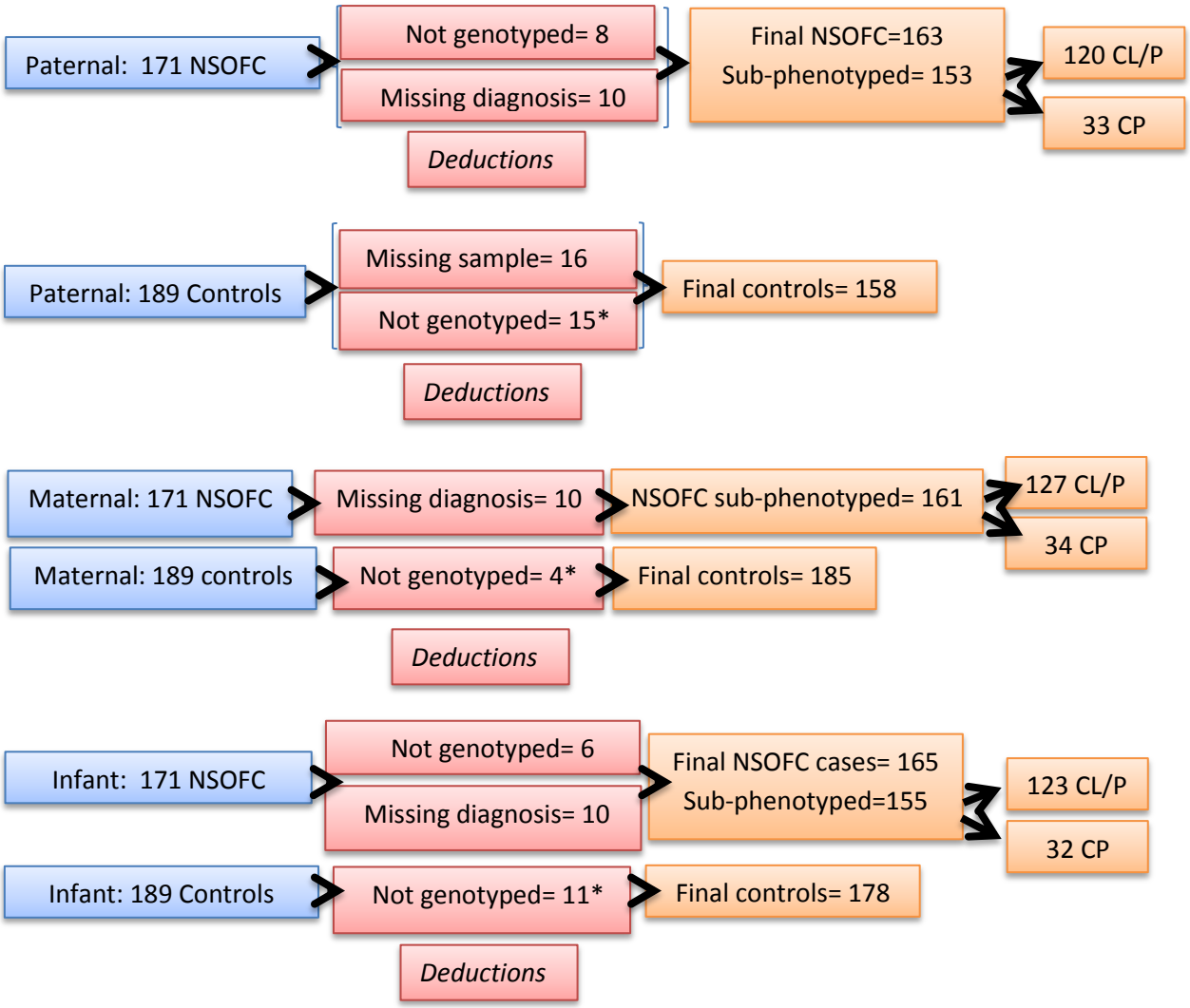
Groups	**Cases**			**Controls**		
Genotype	Common homozygous	Heterozygous	Rare homozygous	Common homozygous	Heterozygous	Rare homozygous
Paternal *IRF6* rs2013162 Cases=163, controls=158						
Observed	70	85	8	81	66	11
Expected	77.65	69.71	15.65	82.25	63.49	12.25
X^2(df), P value	7.85 (2), 0.002**			0.25 (2), 0.25		
Maternal *IRF6* rs2013162 cases=171, controls=185						
Observed	79	79	13	94	63	28
Expected	82	72.76	16.12	85.14	80.73	19.14
X^2(df), P value	1.25(2), 0.536			8.92(2), 0.01**		
Paternal *IRF6* rs2235375 cases=165, controls=169						
Observed	23	84	58	82	68	19
Expected	25.61	78.79	60.6	79.62	72.76	16.62
X^2(df), P value	0.72(2), 0.697			0.72(2), 0.697		
Maternal *IRF6* rs2235375 cases=171, controls=189						
Observed	36	67	68	41	84	64
Expected	28.25	82.51	60.25	36.45	93.1	59.45
X^2(df), P value	6.04 (2), 0.05**			1.81(2), 0.405		
Paternal *IRF6* rs2235371 cases=163, controls=170						
Observed	162	1	0	168	1	1
Expected	161	3	0	167	3	0
X^2(df), P value	1.34 (2) 0.512			1.34 (2), 0.512		
Maternal *IRF6* rs2235371cases=169 controls=189						
Observed	169	0	0	184	5	0
Expected	169	0	0	184	5	0
X^2(df), P value	<0.001(2), 1			<0.001(2), 1		
Paternal *VAX1* rs4752028 cases=165, controls=168						
Observed	95	65	5	134	29	5
Expected	98.5	58	8.5	131.3	34.47	2.26
X^2(df), P value	2.41 (2), 0.3			4.25(2), 0.12		
Maternal *VAX1* rs4752028 cases=168, controls=187						
Observed	104	52	12	151	32	4
Expected	100	58.8	8.6	149	35.7	2.14
X^2(df), P value	2.14 (2), 0.318			2.03 (2), 0.363		

Paternal *VAX1* rs7078160 cases=163, controls=165						
Observed	120	35	8	141	18	6
Expected	116	43	4	135.6	29.7	1.63
X^2(df), P value	5.63(2), 0.06			21.76 (2), <0.001**		
Maternal *VAX1* rs7078160 cases=170, controls=189						
Observed	127	31	12	164	19	6
Expected	119.45	46.1	4.45	159.27	28.46	1.27
X^2(df), P value	18.23 (2), <0.001**			20.9 (2), <0.001**		

**The Chi-square statistic is significant at the 0.05 level

5.5.2 *IRF6* rs2013162 analysis

Out of the 171 cases and 189 controls; 23 (8 cases and 15 controls) paternal samples, four maternal control samples and 17 infant samples (6 cases and 11 controls) did not produce genotyping values for *IRF6* rs2013162. The phenotype diagnosis for ten NSOFC cases are missing. Sixteen control fathers refused to give their saliva. This resulted in; 153 NSOFC (120 CL/P, and 33 CP) paternal genotypes compared to 158 controls; 161 NSOFC (127 CL/P and 34 CP) maternal genotypes compared to 185 controls; and 155 NSOFC (123 CL/P and 32 CP) infant genotypes compared to 178 controls, according to *IRF6* rs2013162genotype variance (Figure 5.2).



* The saliva sample did not produce genotyping values for IRF6 rs2013162

Figure 5.2: Sample flaw description for *IRF6* rs2013162 genotype variance infant-parental triad

5.5.2.1 Transmission Disequilibrium Test (TDT):

Table 5.41 shows the Transmission Disequilibrium Test (TDT) for *IRF6* rs2013162 common and rare alleles using the Family Based Association Test (FBAT) gene analysis among NSOFC, CL/P and CP infant-parental triads. A statistically significant over-transmission of the common allele (C) in NSOFC families including CL/P cases (P= 0.014 and P= 0.018, respectively) was found. PLINK testing also found a significant reduction of NSOFC and CL/P risk with the *IRF6* rs2013162 rare allele (P= 0.016 and OR: 0.667 for NSOFC and P=

0.018 and OR: 0.644 for CL/P). The *IRF6* variant was significantly over-transmitted from the paternal side (P= 0.05) which shows that the significant association observed for *IRF6* rs2013162 was mainly driven from variants coming from the father (Table 5.42).

IRF6 rs2013162 variants did not show an association with CP infant-parental triads following either FBAT or PLINK testing.

5.5.2.2. Comparison between case and control *IRF6* rs2013162 genotypes:

Table 5.43 shows the distribution of *IRF6* rs2013162 infant-parental triad genotypes for NSOFC, CL/P and CP cases compared to controls. There were no significant differences between cases and controls in paternal *IRF6* rs2013162 genotype (P= 0.166 for NSOFC, P= 0.08 for CL/P and P= 0.92 for CP), infant *IRF6* rs2013162 genotype (P= 0.33 for NSOFC, P= 0.18 for CL/P and P= 0.32 for CP) and maternal CP *IRF6* rs2013162 genotype (P= 0.187). However, there were significant differences in maternal *IRF6* rs2013162 genotypes for NSOFC (P= 0.018) and CL/P (P= 0.024) in cases compared to controls.

After chi-square adjustment and Bonferroni correlation was carried out, it was found that the maternal homozygous rare allele genotype (AA) was significantly more prevalent in controls than in NSOFC cases and in CL/P. Furthermore, the heterozygous genotype (CA) was significantly more prevalent in cases than in controls.

5.5.2.3 Comparison between case and control *IRF6* rs2013162 alleles:

Table 5.44 shows the distribution of *IRF6* rs2013162 infant-parental triad allele frequencies in NSOFC, CL/P and CP cases compared to controls. There were no statistical significant differences between the different NSOFC phenotypes and controls.

Table 5.41: Transmission Disequilibrium test (TDT) results for *IRF6* rs2013162 variants among NSOFC infant-parental triads and its sub-phenotypes (CL/P and CP) using FBAT analysis.

Type of NSOFC	Allele	afreq	fam#	SE	Var(S)	Z test	P value
NSOFC	**C**	0.697	104	15.000	37.500	2.449	0.014**
	A	0.303	104	-15.000	37.500	2.449	-0.014**
CL/P	**C**	0.696	79	13	30	2.373	0.018**
	A	0.304	79	-13	30	-2.373	0.018**
CP	**C**	0.623	21	1.5	6.75	0.577	0.564
	A	0.377	21	-1.5	6.75	-0.577	0.564

**Significant at the 0.05 level.

afreq :Estimating allele frequencies

fam#: Number of families

SE: Standard error

Var(S): Value of the average of the squared differences from the mean

Table 5.42: Testing *IRF6* rs2013162 alleles for transmission disequilibrium and parent of origin using PLINK analysis for NSOFC infant-parental triads and its sub-phenotypes (CL/P and CP).

NSOFC	Transmitted/ Untransmitted minor allele	P-value	OR	A:U_PAR	P-value	Combined statistics P-value
NSOFC	58/88	0.016**	0.667	01:02	0.564	0.014**
CL/P	47/73	0.018**	0.644	1:02	0.564	0.015**
CP	12/15	0.564	0.8	00:00	NA	0.257
Parent of origin effect						
	T:U_PAT	Paternal P-value	T:U_MAT	Maternal P-value	POO Z	POO P
NSOFC	29:46:00	0.05**	29:41:00	0.152	-0.339	0.735
CL/P	24.5:39.5	0.061	22.5:33.5	0.142	-0.212	0.832
CP	05:07	0.564	07:08	0.796	-0.26	0.795

A:U PAR: Parental discordance counts
POO: Parents of Origin
T:U PAT: Paternal transmitted: untransmitted counts
T:U MAT: Maternal transmitted: untransmitted counts
**Significant relationship P≤0.05

Table 5.43 Distribution of IRF6 rs2013162 infant-parental triad genotypes according to NSOFC phenotypes (CL/P and CP) and compared to controls. There were missing samples; 23 (8 cases and 15 controls) paternal samples, four maternal control samples and 17 infant samples (6 cases and 11 controls). The phenotype diagnosis of ten NSOFC cases are missing.

Genotype	**NSOFC** (%)	**CL/P** (%)	**CP** (%)	**Control** (%)
Paternal *IRF6* rs2013162 (X^2=5.02, df=4, P= 0.285)				
CC*	70 (42.9)	51 (42.5)	16 (48.5)	81 (51.3)
AA	8 (4.9)	4 (3.3)	2 (6.1)	11 (7)
CA	85 (52.1)	65 (54.2)	15 (45.5)	66 (41.8)
Total	163[C] (100)	120	33 (100)	158(100)
P-value	0.166	0.08	0.92	
Maternal *IRF6* rs2013162 (X^2=9.22, df=4, P= 0.056)				
CC*	79 (46.2)	62 (48.8)	14 (41.2)	94 (50.8)
AA	13 (7.6)	8 (6.3)	3 (8.8)	28 (15.1)
CA	79 (46.2)	57 (44.9)	17 (50)	63 (34.1)
Total	171[C] (100)	127 (100)	34 (100)	185 (100)
P-value	0.018**	0.024**	0.19	
Infant *IRF6* rs2013162 (X^2=5.42, df=4, P= 0.247)				
CC*	86 (52.1)	67 (54.5)	13 (40.6)	95 (53.4)
AA	11 (6.1)	6 (4.9)	3 (9.4)	19 (10.7)
CA	68 (41.2)	50 (40.7)	16 (50)	64 (36)
Total	165[C] (100)	123 (100)	32 (100)	178 (100)
P-value	0.33	0.18	0.32	

* The common homozygous allele genotype

**The Chi-square statistic is significant at the 0.05 level.

[C] Ten NSOFC cases was not sub-phenotyped

Table 5.44 Distribution of *IRF6* rs2013162 infant-parental triad allele frequencies in NSOFC, CL/P and CP cases and compared to controls.

Allele type	**NSOFC** (%)	**CL/P** (%)	**CP** (%)	**Control** (%)
Paternal *IRF6* rs2013162(X^2=0.44, df=2, P= 0.803)				
C*	225 (69)	167 (69.6)	47 (71.2)	228 (72.2)
A	101 (31)	73 (30.4)	19 (28.8)	88 (27.8)
Total	326[C] (100)	240 (100)	66 (100)	316 (100)
P-value	0.38	0.508	0.877	
OR (95% CI)	1.16 (0.83–1.63)	1.13 (0.78–1.64)	1.05 (0.58–1.88)	
Maternal *IRF6* rs2013162(X^2=1.09, df=2, P= 0.58)				
C*	237 (69.7)	181 (71.3)	45 (66.2)	251 (67.1)
A	105 (30.7)	73 (28.7)	23 (33.8)	119 (32.9)
Total	342[C] (100)	254 (100)	68 (100)	370 (100)
P-value	0.675	0.363	0.788	
OR (95% CI)	0.93 (0.68–1.28)	1.18 (0.8–1.67)	1.08 (0.62–1.86)	
Infants IRF6 rs2013162(X^2=2.32, df=2, P= 0.313)				
C*	240 (72.7)	184 (74.8)	42 (65.6)	254 (71.3)
A	90 (27.3)	62 (25.2)	22 (34.4)	102 (28.7)
Total	330[C] (100)	246 (100)	64 (100)	356 (100)
P-value	0.688	0.26	0.425	
OR (95% CI)	1.07 (0.77–1.5)	0.84 (0.58–1.21)	1.30 (0.74–2.29)	

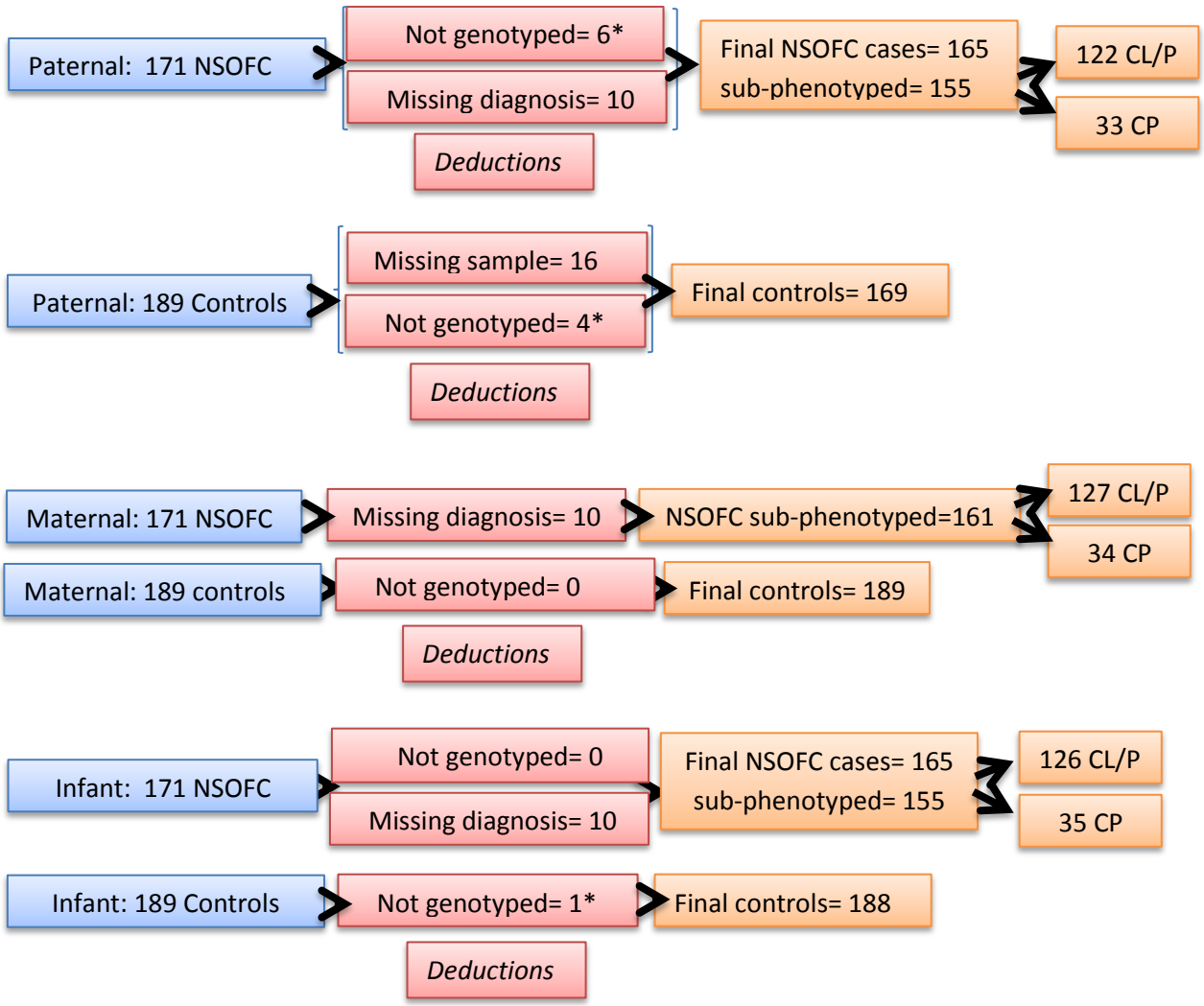
* The common homozygous allele genotype

[C] Ten NSOFC cases were not sub-phenotyped

5.5.3 *IRF6* rs2235375 analysis

Out of the total 171 cases and 189 controls; 10 (6 cases and 4 controls) paternal samples, and one infant control did not produce genotyping values for *IRF6* rs2235375. The phenotype diagnosis for ten NSOFC cases are missing. This resulted in; 122 CL/P, and 33 CP paternal genotype compared to 169 controls; 127 CL/P and 34 CP maternal genotype compared to 189

controls; and 126 CL/P and 35 CP infant genotype compared to 188 controls, according to *IRF6* rs2235375 genotype variance (Figure 5.3).



* The saliva sample did not produce genotyping values for IRF6 rs2013162

Figure 5.3: Sample flaw description *IRF6* rs2235375 genotype variance infant-parental triad

5.5.3.1 TDT gene analysis:

Tables 5.45 and 5.46 show the TDT analyses using the FBAT and PLINK tests for *IRF6* rs2235375 common and rare alleles. No significant over-transmission of the *IRF6* rs2235375 rare allele in NSOFC families (FBAT: P= 0.61 for NSOFC; P= 0.441 for CL/P; and P= 0.577 for CP. For PLINK: P= 0.667 for NSOFC; P= 0.441 for CL/P; and P= 0.578 for CP).

5.5.3.2 Comparison between case and control *IRF6* rs2235375 genotypes:

Table 5.47 shows the distribution of *IRF6* rs2235375 genotypes in NSOFC, CL/P and CP cases compared to controls in infant-parental triads. There was a statistically significant difference found between paternal genotypes of NSOFC infants and controls (P= 0.047). After chi-square adjustment using Bonferroni correction for the *IRF6* rs2235375 relationship with NSOFC, the homozygous rare genotype (GG) was found to be significantly more frequent in fathers of the controls than in fathers of cases with NSOFC. However, no statistically significant differences were found between cases and controls in mothers or infants (P= 0.14 and P= 0.18, respectively).

5.5.3.3 Comparison between case and control *IRF6* rs2235375 alleles:

Table 5.48 shows the frequency of *IRF6* rs2235375 alleles in case and control infant-parental triads. There was no statistical significant difference found between cases compared to controls in infant-parental triads for this SNP except for paternal rare allele (G) which shows a statistical significant lower frequency of NSOFC cases compared to control (P= 0.03, OR:0.7 and 95% CI: 0.5 to 0.93).

Table 5.45: Transmission Disequilibrium test (TDT) results for *IRF6* rs2235375 variants among NSOFC infant-parental triads and its sub-phenotypes (CL/P and CP) using FBAT analysis.

Type of NSOFC	**Allele**	**afreq**	**fam#**	**SE**	**Var(S)**	**Z test**	**P value**
NSOFC	C*	0.360	91	-3.000	34.500	-0.511	0.61
	G	0.640	91	3.000	34.500	0.511	0.61
CL/P	C*	0.634	69	4	27	0.77	0.441
	G	0.366	69	-4	27	-0.77	0.441
CP	C*	0.636	20	-1.5	7.250	-0.557	0.577
	G	0.364	20	1.5	7.250	0.557	0.577

*The common allele

afreq :Estimating allele frequencies

fam#: Number of families

SE: Standard error

Var(S): Value of the average of the squared differences from the mean

Table 5.46: Testing *IRF6* rs2235375 alleles for transmission disequilibrium and parent of origin using PLINK analysis for NSOFC infant-parental triads and sub-phenotypes (CL/P and CP).

NSOFC	**Transmitted/ Untransmitted minor allele**	**P-value**	**OR**	**A:U_PAR**	**P-value**	**Combined statistics P-value**
NSOFC	65/70	0.667	0.929	00:00	NA	0.667
CL/P	50/58	0.441	0.862	00:00	NA	0.441
CP	16/13	0.578	1.23	00:00	NA	0.257
Parent of origin effect						
	T:U_PAT	Paternal P-value	T:U_MAT	Maternal P-value	POO Z	POO P
NSOFC	37:40:00	0.732	28:30:00	0.793	-0.026	0.979
CL/P	28.5:34.5	0.45	21.5:23.5	0.766	-0.261	0.794
CP	09:06	0.439	07:07	1	-0.54	0.595

A:U PAR: Parental discordance counts

POO: Parents of Origin

T:U PAT: Paternal transmitted: untransmitted counts

T:U MAT: Maternal transmitted: untransmitted counts

**Significant relationship

Table 5.47: Distribution of *IRF6* rs2235375 infant-parental triad genotypes according to NSOFC phenotypes (CL/P and CP) and compared to control infant-parental triads. Ten (6 cases and 4 controls) paternal samples, and one infant control did not produce genotyping values for *IRF6* rs2235375.

Genotype	**NSOFC** (%)	**CL/P** (%)	**CP** (%)	**Control** (%)
Paternal *IRF6* rs2235375(X^2=6.04, df=4, P= 0.196)				
CC*	23 (13.9)	16 (13.1)	3 (9.1)	19 (11.2)
GG	58 (35.2)	45 (36.9)	11 (33.3)	82 (48.5)
CG	84 (50.9)	61 (50)	19 (57.6)	68 (40.2)
Total	165[C] (100)	122 (100)	33 (100)	169 (100)
P-value	0.047*	0.14	0.18	
Maternal *IRF6* rs2235375 (X^2=2.2, df=4, P= 0.**7)**				
CC*	36 (21.1)	25 (19.7)	8 (23.5)	41 (21.7)
GG	68 (39.8)	54 (42.5)	11 (32.4)	84 (44.4)
CG	67 (39.2)	48 (37.8)	15 (44.1)	64 (33.9)
Total	171[C] (100)	127 (100)	34 (100)	189 (100)
P-value	0.554	0.76	0.39	
Infant *IRF6* rs2235375 (X^2=1.01, df=4, P= 0.908)				
CC*	34 (19.9)	26 (20.6)	7 (20)	38 (20.2)
GG	77 (45)	57 (45.2)	13 (37.1)	83 (44.1)
CG	60 (35.1)	43 (34.1)	15 (42.9)	67 (35.6)
Total	171[C] (100)	126 (100)	35 (100)	188 (100)
P-value	0.99	0.96	0.69	

* The common homozygous allele genotype

**The Chi-square statistic is significant at the 0.05 level.

[C] Ten NSOFC cases were not sub-phenotyped

Table 5.48: Distribution of *IRF6* rs2235375 infant-parental triad allele frequencies in NSOFC, CL/P and CP cases and compared to controls.

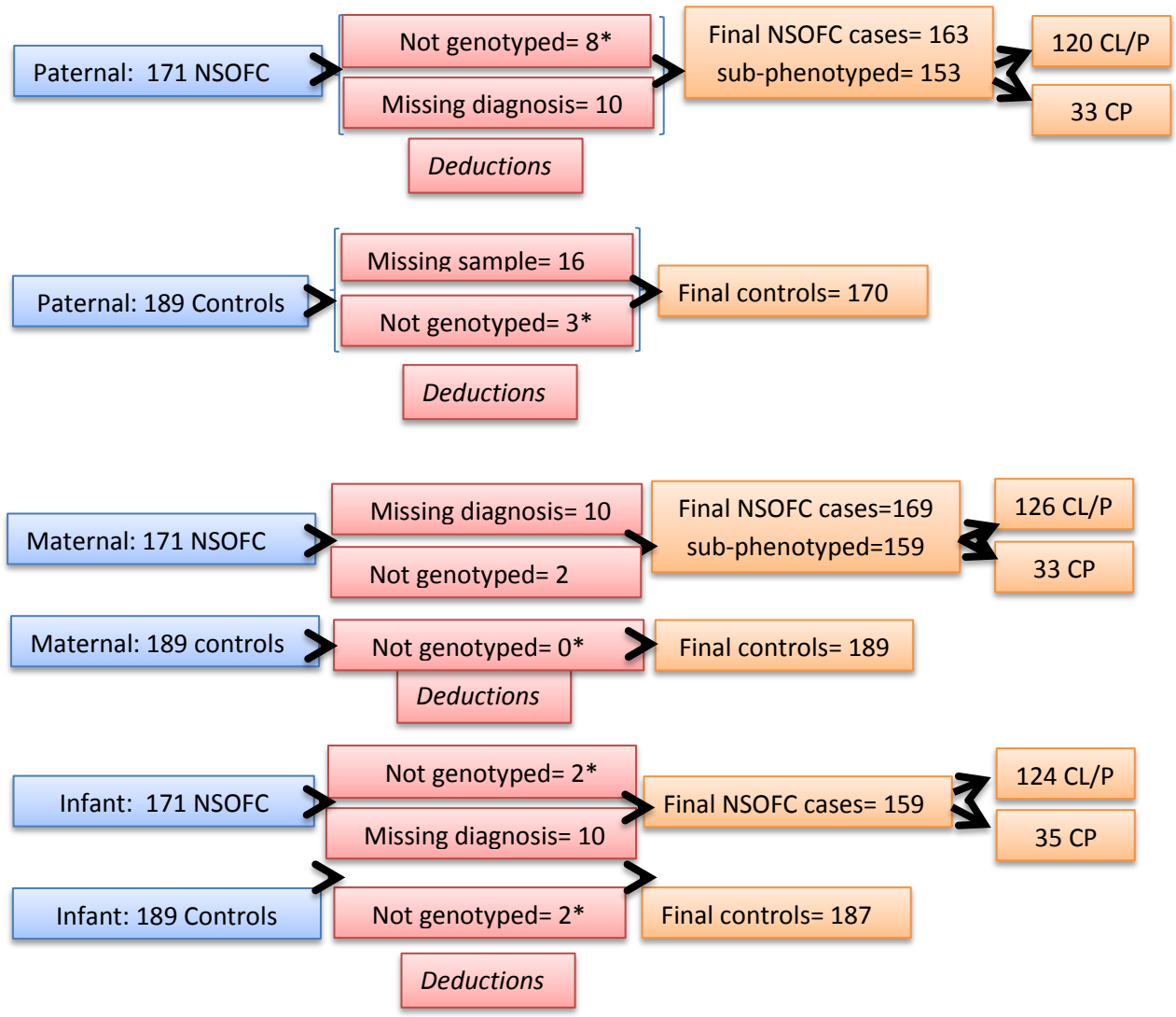
Allele type	**NSOFC** (%)	**CL/P** (%)	**CP** (%)	**Control** (%)
Paternal *IRF6* rs2235375(X^2=3.21, df=2, P= 0.2)				
C*	130 (41.2)	93 (38.1)	25 (37.9)	106 (31.4)
G	200 (38.8)	151 (61.9)	41 (62.1)	232 (68.6)
Total	330 (100)	244 (100)	66 (100)	338 (100)
P-value	0.03**	0.09	0.301	
OR (95% CI)	0.7 (0.5 -0.97)	1.35 (0.95 - 1.90)	1.33 (0.77 - 2.31)	
Maternal *IRF6* rs2235375(X^2=1.25, df=2, P= 0.534)				
C*	139 (40.6)	98 (38.6)	31 (45.6)	146 (38.6)
G	203 (59.4)	156 (61.4)	37 (44.4)	232 (61.4)
Total	342 (100)	254 (100)	68 (100)	378 (100)
P-value	0.58	1	0.28	
OR (95% CI)	1.09 (0.81 - 1.47)	1.00 (0.72 - 1.38)	1.33 (0.79 - 2.24)	
Infant *IRF6* rs2235375 (X^2=0.46, df=2, P= 0.793)				
C*	128 (37.4)	95 (37.7)	29 (41.4)	143 (37.1)
G	214 (62.6)	157 (62.3)	41 (58.6)	242 (62.9)
Total	342 (100)	252 (100)	70 (100)	385 (100)
P value	0.916	0.867	0.5	
OR-(95% CI)	0.97 (0.72 - 1.32)	0.99 (0.71 - 1.37)	0.84 (0.5 - 1.4)	

**The Chi-square statistic is significant at the 0.05 level.

5.5.4 *IRF6* rs2235371 analysis

Out of the total 171 cases and 189 controls; 11 (8 cases and 3 controls) paternal samples, two maternal NSOFC samples and four infant samples (two cases and two controls) did not produce genotyping values for *IRF6* rs2235371. The phenotype diagnosis for ten NSOFC

cases are missing. This resulted in; 120 CL/P, and 33 CP paternal genotype compared to 170 controls; 126 CL/P and 33 CP maternal genotype compared to 189 controls; and 124 CL/P and 35 CP infant genotype compared to 187 controls, according to *IRF6* rs2235371 genotype variance (Figure 5.4).



* The saliva sample did not produce genotyping values for IRF6 rs2013162

Figure 5.4: Sample flaw description for *IRF6* rs2235371 genotype variance infant-parental triad

5.5.4.1 TDT gene analysis:

Table 5.49 shows the frequency of *IRF6* rs2235371 genotypes for case and control infant-parental triads. Almost all cases and controls were homozygous for the common allele (CC) (99.4% and 98.8% respectively). Therefore TDT using FBAT and PLINK analyses was not carried out as the number of rare alleles was negligible.

5.5.4.2 Comparison between case and control *IRF6* rs2235371 genotypes:

There was no statistically significant differences between *IRF6* rs2235371 genotypes in NSOFC families compared with controls even after chi square correction except in mothers where there were 5 (2.5%) maternal controls with a heterozygous genotype (CT) compared to zero NSOFC mothers with the CT genotype (P= 0.03) (table 5.49).

5.5.4.3 Comparison between case and control *IRF6* rs2235371 alleles:

Table 5.51 shows the frequency of *IRF6* rs2235371 alleles in case and control infant-parental triads. There were significantly more controls with the *IRF6* rs2235371 rare allele (T) compared to NSOFC (P= 0.002, OR: 0.05 and 95% CI: 0.0 to 0.86) (Table 5. 50).

Table 5.49: Distribution of *IRF6* rs2235371 infant-parental triad genotypes according to NSOFC phenotypes (CL/P and CP) and compared to controls. Eleven (8 cases and 3 controls) paternal samples, two maternal NSOFC samples and four infant samples (two cases and two controls) did not produce genotyping values for *IRF6* rs2235371. The phenotype diagnosis for ten NSOFC cases are missing

Genotype	**NSOFC** (%)	**CL/P** (%)	**CP** (%)	**Control** (%)
Paternal *IRF6* rs2235371 (X^2=1.2, df=4, P= 0.878**)**				
CC*	162 (99.4)	119 (99.2)	33 (100)	168 (98.8)
TT	0	0	0	1 (0.6)
CT	1 (0.6)	1 (0.8)	33 (100)	1 (0.6)
Total	163[C] (100)	120	33 (100)	170 (100)
P-value	0.62	0.68	0.82	
Maternal *IRF6* rs2235371(X^2=4.27, df=2, P= 0.118)				
CC*	169 (100)	126 (100)	33 (100)	184 (97.4)
TT	0	0	0	0
CT	0	0	0	5 (2.6)
Total	169[C] (100)	126 (100)	33 (100)	189 (100)
P-value	0.03**	0.066	0.35	
Infant *IRF6* rs2235371(X^2=2.57, df=2, P= 0.276**)**				
CC*	169 (100)	124 (100)	35 (100)	184 (98.4)
TT	0	0	0	3 (1.6)
CT	0	0	0	0
Total	169[C] (100)	124 (100)	35 (100)	187 (100)
P-value	0.1	0.16	0.32	

*The common homozygous allele genotype

**The Chi-square statistic is significant at the 0.05 level

[C] Ten NSOFC cases were not sub-phenotyped.

Table 5.50: Distribution of *IRF6* rs2235371 infant-parental triad allele frequencies in NSOFC, CL/P and CP cases and compared to controls.

Allele type	**NSOFC** (%)	**CL/P** (%)	**CP** (%)	**Control** (%)
Paternal *IRF6* rs2235371(X^2=10.95, df=2, P= 0.621				
C	325 (99.7)	239 (99.6)	66 (100)	337 (99.1)
T	1 (0.3)	1 (0.4)	0	3 (0.9)
Total	326[C] (100)	240 (100)	66 (100)	340 (100)
P-value	0.336	0.505	0.444	
OR (95% CI)	0.35 (0.04 - 3.34)	0.47 (0.05 - 4.55)	a	
Maternal *IRF6* rs2235371(X^2=8.54, df=2, P= 0.014**)				
C	338 (100)	252 (100)	66 (100)	368 (97.4)
T	0	0	0	10 (2.6)
Total	338[C] (100)	252 (100)	66 (100)	378 (100)
P-value	0.002**	0.009**	0.18	
OR (95% CI)	a	a	a	
Infant *IRF6* rs2235371(X^2=3.53, df=2, P= 0.171)				
C	338 (100)	248 (100)	70 (100)	368 (98.4)
T	0	0	0	6 (1.6)
Total	338[C] (100)	248 (100)	70 (100)	374 (100)
P-value	0.019**	0.045**	0.3	
OR (95% CI)	a	a	a	

**The Chi-square statistic is significant at the 0.05 level.

a. Not possible to analyse because the groups contain zero values

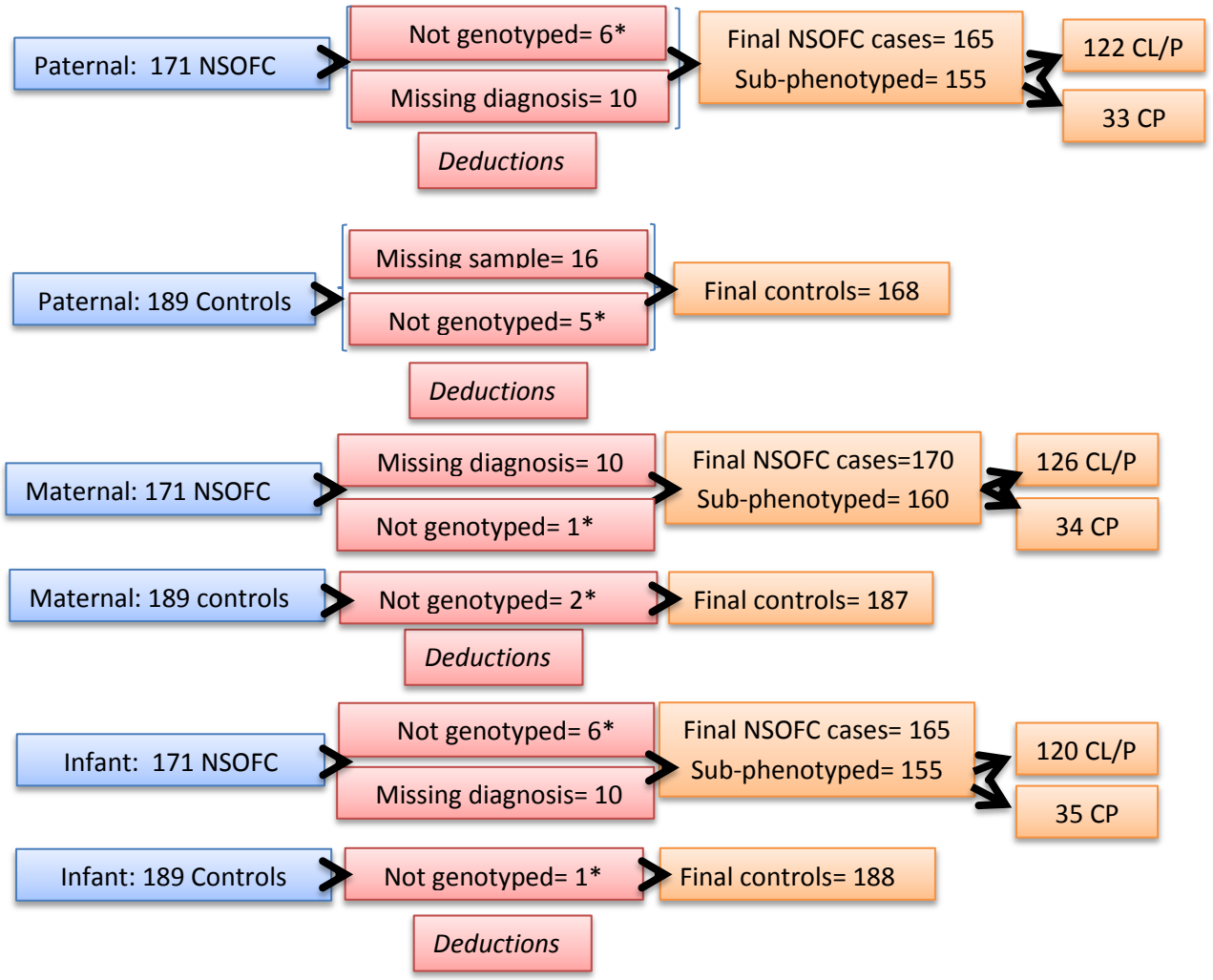
If one of the cells contains zero value, the OR and 95% CI are not calculated

[C] Ten NSOFC cases were not sub-phenotyped.

5.5.5 *VAX1* rs4752028 analysis

In addition to the 10 NSOFC cases with missing phenotype diagnosis out of the 171 NSOFC cases and 189 controls; 11 (6 cases and 5 controls) paternal samples, 3 (one case and two

controls) maternal samples and 7 infant samples (6 cases and one controls) did not produce genotyping values for *VAX1* rs4752028. This resulted in; 122 CL/P, and 33 CP paternal genotype compared to 168 controls; 126 CL/P and 34 CP maternal genotype compared to 187 controls; and 120 CL/P and 35 CP infant genotype compared to 188 controls, according to *VAX1* rs4752028 genotype variance (Figure 5.5)



* The saliva sample did not produce genotyping values for IRF6 rs2013162

Figure 5.5: Sample flaw description for *VAX1* rs4752028 genotype variance infant-parental triad

5.5.5.1 TDT gene analysis:

Tables 5.51 and 5.52 shows the TDT analyses for *VAX1* rs4752028 using FBAT and PLINK tests. No statistically significant over-transmission of the rare allele (C) was found in NSOFC, CL/P or CP families (FBAT: P= 0.769 for NSOFC; P= 0.651 for CL/P; and P= 1 for CP. For PLINK: P= 1 for NSOFC; P= 0.651 for CL/P; and P= 686 for CP).

5.5.5.2 Comparison between case and control *VAX1* rs4752028 genotypes:

Table 5.53 shows the distribution of *VAX1* rs4752028 genotypes in case and control infant-parental triads. There was a statistically significant difference between case and control *VAX1* rs4752028 genotype in infant-parental triads for NSOFC, CL/P and CP cases: P< 0.001 for NSOFC infant-parental triads and infant CL/P; P= 0.001 for maternal and paternal CL/P and paternal CP; and P= 0.02 for infant CP.

After chi square adjustment using Bonferroni correlation in infant-parental trios, it was found that in fathers; the homozygous common allele genotype (TT) was found significantly more often in controls than in cases for NSOFC, CL/P and CP ($P \leq 0.05$). Furthermore, the heterozygous genotype (CT) was significantly more prevalent in cases than in controls for the different cleft phenotypes. For mothers and infants, the homozygous rare allele genotype (CC) occurred significantly more often in cases than in controls for NSOFC and the CL/P sub-phenotype; the homozygous common allele (TT) was significantly found more often in controls for NSOFC and CL/P; the heterozygous genotype (CT) was significantly present more in NSOFC and its sub-phenotypes except in mothers of CP infants.

5.5.5.3 Comparison between case and control *VAX1* rs4752028 alleles:

Table 5.54 shows the frequency of the *VAX1* rs4752028 rare allele in case and control infant-parental triads for NSOFC, CL/P and CP. In all comparisons the rare allele was found

significantly more often in cases than in controls and was associated with: NSOFC (fathers: P< 0.001, OR: 2.24 95% CI: 1.47 to 3.4; mothers: P< 0.001, OR: 2.40, 95% CI: 1.61 to 3.7; and infants: P< 0.001, OR: 2.71, 95% CI: 1.78 to 4.13); CL/P (fathers: P= 0.001, OR: 2.16 and 95% CI: 1.38 to 3.4; mothers: P< 0.001, OR:2.39, 95% CI: 1.53 to 3.71; and infants with P< 0.001, OR: 2.77, and 95% CI: 1.77 to 4.34); and CP (fathers: P= 0.015, OR: 2.24 and 95% CI: 1.15 to 4.36; mothers: P= 0.049, OR: 1.97 and 95% CI: 0.99 to 3.93; and infants: P= 0.009, OR: 2.43 and 95% CI: 1.25 to 4.7).

Table 5.51: Transmission Disequilibrium test (TDT) results for *VAX1* rs4752028 variants among NSOFC infant parental triads and its sub-phenotypes (CL/P and CP) using FBAT analysis.

Type of NSOFC	**Allele**	**afreq**	**fam#**	**SE**	**Var(S)**	**Z test**	**P value**
NSOFC	**T***	0.785	71	-1.500	.250	-0.293	0.769
	C	0.215	71	1.500	26.250	0.293	0.769
CL/P	**T***	0.767	53	-2	19.5	-0.453	0.651
	C	0.233	53	2	19.5	0.453	0.651
CP	**T***	0.779	14	0	5.5	0	1
	C	0.221	14	0	5.5	0	1

* The common allele

afreq :Estimating allele frequencies

fam#: Number of families

SE: Standard error

Var(S): Value of the average of the squared differences from the mean

Table 5.52: Testing *VAX1* rs4752028 for transmission disequilibrium and parent of origin using PLINK analysis for NSOFC infant-parental triads and its sub-phenotypes (CL/P and CP).

Transmission disequilibrium Test						
NSOFC	**Transmitted/ Untransmitted minor allele**	**P-value**	**OR**	**A:U_PAR**	**P-value**	**Combined statistics P-value**
NSOFC	51/47	0.686	1.09	01:01	1	0.689
CL/P	41/37	0.651	1.11	01:01	1	0.655
CP	11/11	1	1	00:00	NA	1
Parent of origin effect						
	T:U_PAT	**Paternal P-value**	**T:U_MAT**	**Maternal P-value**	**POO Z**	**POO P**
NSOFC	29:27:00	0.789	22:20	0.757	-0.058	0.954
CL/P	22.5:20.5	0.76	18.5:16.5	0.735	-0.047	0.962
CP	07:07	1	04:04	1	0	1

A:U PAR: Parental discordance counts

POO: Parents of Origin

T:U PAT: Paternal transmitted: untransmitted counts

T:U MAT: Maternal transmitted: untransmitted counts

Table 5.53: Distribution of *VAX1* rs4752028 infant-parental triad genotypes according to NSOFC phenotypes (CL/P and CP) and compared to controls. Eleven (6 cases and 5 controls) paternal samples, 5 (3 cases and two controls) maternal samples and 7 infant samples (6 cases and one controls) did not produce genotyping values for *VAX1* rs4752028. The phenotype diagnosis for ten NSOFC cases are missing

Genotype	**NSOFC** (%)	**CL/P** (%)	**CP** (%)	**Control** (%)
Paternal *VAX1* rs4752028 X^2=20.31, df=4, P<0.001**				
TT*	95 (57.6)	73 (59.8)	18 (54.5)	134 (79.8)
CC	5 (3)	5 (4.1)	0	5 (2.9)
CT	65 (39.4)	44 (36.1)	15 (45.5)	29 (17.3)
Total	165 [C] (100)	122 (100)	33 (100)	168(100)
P-value	<0.001**	0.001**	0.001**	
Maternal *VAX1* rs4752028 X^2=13.61, df=4, P= 0.009				
TT*	106 (62.4)	80 (63.5)	23 (67.6)	151 (80.7)
CC	12 (7.1)	10 (7.9)	2 (5.9)	4 (2.1)
CT	52 (30.6)	36 (28.6)	9 (26.5)	32 (17.1)
Total	170[C] (100)	126 (100)	34 (100)	187(100)
P-value	0.000**	0.001**	0.18	
Infant *VAX1* rs4752028 X^2=21.96, df=4, P<0.001****				
TT*	98 (59.4)	72 (60)	21 (60)	153 (81.4)
CC	10 (6.1)	9 (7.5)	1 (2.9)	3 (1.6)
CT	57 (34.5)	39 (32.5)	13 (37.1)	32 (17)
Total	165[C] (100)	120 (100)	35 (100)	188 (100)
P-value	<0.001**	<0.001**	0.02**	

* The homozygous common allele genotype

**The Chi-square statistic is significant at the 0.05 level.

[C] Ten NSOFC cases were not sub-phenotyped

Table 5.54: Distribution of *VAX1* rs4752028 infant-parental triad allele frequencies in NSOFC, CL/P and CP cases compared to controls.

Allele type	**NSOFC** (%)	**CL/P** (%)	**CP** (%)	**Control** (%)
Paternal *VAX1* rs4752028 (X^2=13.15, df=2, P= 0.002**)				
T*	255 (77.3)	190 (77.9)	51 (77.3)	297 (87.9)
C	75 (22.7)	54 (22.1)	15 (22.7)	39 (2.9)
Total	330[C] (100)	244 (100)	66 (100)	336 (100)
P-value	<0.001**	0.001**	0.015**	
OR (95% CI)	2.24 (1.47 - 3.41)	2.16 (1.38 - 3.40)	2.24 (1.15 - 4.36)	
Maternal *VAX1* rs4752028 (X^2=15.77, df=2, P<0.001**)				
T*	264 (76.5)	196 (77.7)	55 (80.8)	334 (89.3)
C	76 (23.5)	56 (22.2)	13 (19.1)	40 (10.7)
Total	400[C] (100)	252 (100)	68 (100)	374 (100)
P-value	<0.001**	<0.001**	0.049	
OR (95% CI)	2.44 (1.61 - 3.7)	2.39 (1.53 - 3.71)	1.97 (0.99 - 3.93)	
Infant *VAX1* rs4752028(X^2=21.94, df=2, P<0.001**)**				
T*	253 (76.7)	183 (68.8)	55 (78.6)	338 (89.9)
C	77 (23.3)	57 (31.2)	15 (21.4)	38 (10.1)
Total	330[C] (100)	240 (100)	70 (100)	376 (100)
P-value	<0.001**	<0.001**	0.009**	
OR (95% CI)	2.71 (1.78 - 4.13)	2.77 (1.77 – 4.34)	2.43 (1.25 - 4.7)	

* The homozygous common allele.

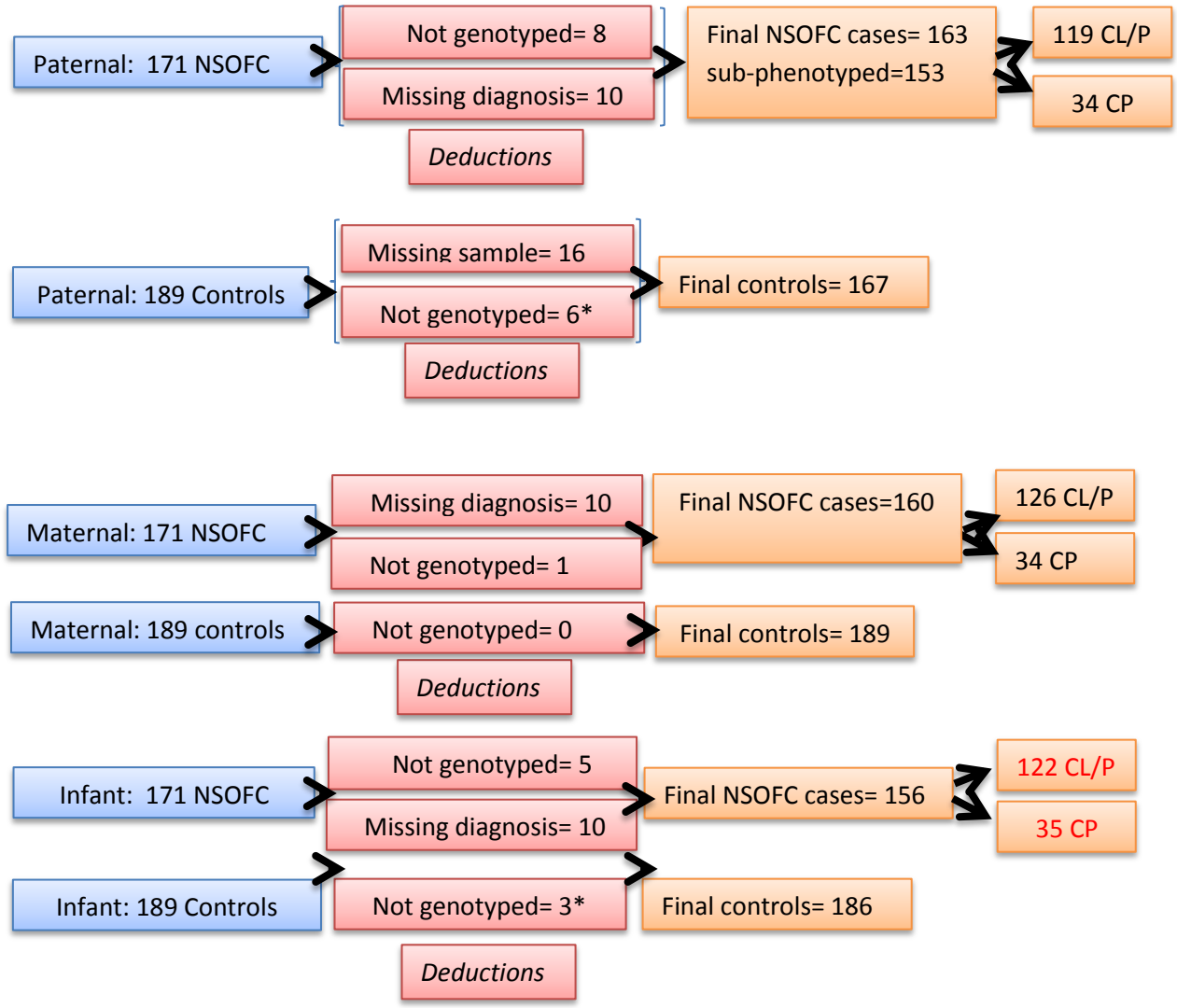
**The Chi-square statistic is significant at the 0.05 level.

[C] Ten NSOFC cases were not sub-phenotyped.

5.5.6 VAX1 rs7078160 analysis

Fourteen (8 cases and 6 controls) paternal samples, one maternal samples and 7 infant samples (5 cases and 3 controls) did not produce genotyping values for IRF6 rs2013162. The phenotype diagnosis for ten NSOFC cases are missing. This resulted in; 119 CL/P, and 34 CP

paternal genotype compared to 167 controls; 126 CL/P and 34 CP maternal genotype compared to 189 controls; and 122CL/P and 35 CP infant genotype compared to 186 controls, according to *VAX1* rs2013162 genotype variance.



* The saliva sample did not produce genotyping values for IRF6 rs2013162

Figure 5.6: Sample flaw description for VAX1 rs7078160 genotype variance infant-parental triad

5.5.6.1 TDT gene analysis:

Tables 5.55 and 5.56 show TDT for *VAX1* rs7078160 using FBAT and PLINK analyses. No statistically significant over-transmission of the rare allele was found in NSOFC and CL/P

families (FBAT: P= 0.293 for NSOFC; and P= 0.327 for CL/P. For PLINK: P= 0.233 for NSOFC; P= 0.327 for CL/P; and P= 0.257 for CP). For CP, the number of included heterozygous alleles was not enough to produce a P-value in FBAT analysis.

5.5.6.2 Comparison between case and control *VAX1* rs7078160 genotypes:

Table 5.57 shows the distribution of *VAX1* rs7078160 genotype frequencies between case and control infant-parental triads. Fathers of NSOFC infants genotypes were significantly different than controls (P= 0.03). Mothers and infants had significant differences between cases and controls for NSOFC (P= 0.02 and P= 0.04, respectively) and CL/P phenotypes (P= 0.004 and P= 0.003, respectively).

After chi square adjustment using Bonferroni correction, the homozygous genotype rare allele (AA) was found to be significantly more frequent in NSOFC and CL/P infants compared to controls ($P \leq 0.05$). The homozygous genotype common allele (GG) was significantly more frequent in infant-parental control triads than in NSOFC cases ($P < 0.05$). The heterozygous genotype (AG) was significantly more frequent in parental controls compared to parents of NSOFC and CL/P infants ($P < 0.05$).

5.5.6.3 Comparison between case and control *VAX1* rs7078160 genotype alleles:

Table 5.58 shows the frequency of alleles in case and control infant-parental triads. In infant-parental triads, there were statistically significant differences between cases and controls in: NSOFC (fathers: P= 0.026, OR: 1.69 and 95% CI: 1.06 to 2.70; mothers: P= 0.001, OR: 2.16 and 95% CI: 1.35 to 3.45; and infants: P= 0.021, OR: 1.74 and 95% CI: 1.08 to 2.80); and CL/P (fathers: P= 0.03, OR: 1.73 and 95% CI: 1.05 to 2.86; mothers: P< 0.001, OR: 2.43 and 95% CI: 1.49 to 3.97; and infants: P<0.001, OR: 2.34 and 95% CI: 1.44 to 3.81) with significantly greater frequency of the rare allele in cases compared to controls.

Table 5.55: Transmission Disequilibrium test (TDT) results for *VAX1* rs7078160 variants among NSOFC infant parental triads and its sub-phenotypes (CL/P and CP) using FBAT analysis.

Type of NSOFC	**Allele**	**afreq**	**fam#**	**SE**	**Var(S)**	**Z test**	**P value**
NSOFC	**G***	0.881	50	4.0	14.500	1.050	0.293
	A	0.119	50	-4.0	14.500	-1.050	0.293
CL/P	**G***	0.872	45	3.5	12.75	0.98	0.327
	A	0.128	45	-3.5	12.75	-0.98	0.327

* The common allele

afreq :Estimating allele frequencies

fam#: Number of families

SE: Standard error

Var(S): Value of the average of the squared differences from the mean

Table 5.56: Testing *VAX1* rs7078160 for transmission disequilibrium and parent of origin using PLINK analysis for NSOFC infant-parental triads and its sub-phenotypes (CL/P and CP).

NSOFC	**Transmitted/ Untransmitted minor allele**	**P-value**	**OR**	**A:U_PAR**	**P-value**	**Combined statistics P-value**
NSOFC	24/33	0.233	0.727	01:02	0.563	0.302
CL/P	22/29	0.327	0.759	02:01	0.564	0.414
CP	2/5	0.257	0.4	00:00	NA	0.257
Parent of origin effect						
	T:U_PAT	Paternal P-value	T:U_MAT	Maternal P-value	POO Z	POO P
NSOFC	11.5:17.5	0.265	12.5:15.5	0.571	-0.381	0.703
CL/P	10:15	0.317	12:14	0.695	-0.443	0.658
CP	1.5:2.5	0.617	0.5:2.5	1	0.59	0.555

A:U PAR: Parental discordance counts

POO: Parent of Origin

T:U PAT: Paternal transmitted: untransmitted counts

T:U MAT: Maternal transmitted: untransmitted counts

**Significant relationship

Table 5.57: Distribution of *VAX1* rs7078160 infant-parental triad genotypes in NSOFC, CL/P and CP cases compared to controls. Fourteen (8 cases and 6 controls) paternal samples, one maternal samples and 7 infant samples (5cases and 3 controls) did not produce genotyping values for *VAX1* rs7078160. The phenotype diagnosis for ten NSOFC cases are missing

Genotype	**NSOFC** (%)	**CL/P** (%)	**CP** (%)	**Control** (%)
Paternal *VAX1* rs7078160 (X^2=1021, df=4, P= 0.037**)				
GG*	120 (73.6)	86 (72.3)	26 (76.5)	141 (84.4)
AA	8 (4.9)	5 (4.2)	3 (8.8)	7 (4.2)
AG	35 (21.5)	28 (23.5)	5 (14.7)	19 (11.4)
Total	163[C] (100)	119 (100)	34 (100)	167 (100)
P-value	0.03**	0.15	0.32	
Maternal *VAX1* rs7078160 (X^2=12.21, df=4, P= 0.016**)				
GG*	127 (74.7)	90 (71.4)	29 (85.3)	164 (86.8)
AA	12 (7.1)	9 (7.1)	2 (5.9)	6 (3.2)
AG	31(18.2)	27 (21.4)	3 (8.8)	19 (10.1)
Total	170[C] (100)	126 (100)	34 (100)	189 (100)
P-value	0.02**	0.004**	0.73	
Infant *VAX1* rs7078160 (X^2=15.7, df=4, P= 0.003)				
GG*	132 (79)	90 (73.8)	32 (91.4)	157 (84.4)
AA	12 (7.2)	12 (9.8)	0	3 (1.6)
AG	23 (13.8)	20 (16.4)	3 (8.6)	26 (14)
Total	167[C] (100)	122 (100)	35 (100)	186 (100)
P-value	0.04**	0.003**	0.49	

* The common allele

**The Chi-square statistic is significant at the 0.05 level

[C] Ten NSOFC cases were not sub-phenotyped

Table 5.58: Distribution of *VAX1* rs7078160 infant-parental triad allele frequencies in NSOFC, CL/P and CP cases compared to controls.

Allele type	NSOFC (%)	CL/P (%)	CP (%)	Control (%)
Paternal *VAX1* rs7078160 (X^2=5.38, df=2, P= 0.068)				
G*	275 (84.4)	200 (84)	57 (83.8)	301 (90.1)
A	51(15.6)	38 (16)	11(16.2)	33 (9.9)
Total	326[C] (100)	238 (100)	68 (100)	334 (100)
P-value	0.026**	0.03**	0.129	
OR (95% CI)	1.69 (1.06 - 2.70)	1.73 (1.05 - 2.86)	1.76 (0.84 - 3.68)	
Maternal *VAX1* rs7078160 (X^2=13.64, df=2, P= 0.001**)				
G*	285 (83.8)	207 (82.1)	61 (89.7)	347 (87.8)
A	55 (16.2)	45 (17.9)	7 (10.3)	31 (8.2)
Total	340[C] (100)	252 (100)	68 (100)	378 (100)
P-value	0.001**	<0.001**	0.569	
OR (95% CI)	2.16 (1.35 - 3.45)	2.43 (1.49 - 3.97)	1.28 (0.54 - 3.05)	
Infants *VAX1* rs7078160 (X^2=16.86, df=2, P<0.001**)				
G*	287 (85.9)	200 (82)	67 (95.7)	340 (91.4)
A	47 (14.1)	44 (18)	3 (8.6)	32 (1.6)
Total	334[C] (100)	244 (100)	70 (100)	372 (100)
P-value	0.021**	0.000**	0.23	
OR (95% CI)	1.74 (1.08 - 2.80)	2.34 (1.44 - 3.81)	0.48 (0.14-1.6)	

* The common allele
**The Chi-square statistic is significant at the 0.05 level.

[C] Ten NSOFC cases were not sub-phenotyped.

5.5.7 Infant *VAX1* genotype variants and paternal consanguinity as risk factors for NSOFC

VAX1 gene (rs4752028 and rs7078160) variances among CL/P and CP infants were compared to controls according to paternal consanguinity.

5.5.7. 1 Infant *VAX1* rs4752028

The distribution of the included sample (cases and control together) according to parental consanguinity and its relationship to infant *VAX1* rs4752028 genotype is presented in Appendix A31. It showed a statistically significant differences between the three infant *VAX1* rs4752028 genotypes according to parental consanguinity (P= 0.04). The prevalence of the infant homozygous rare allele and heterozygous genotypes (CC and CT, respectively) were significantly higher in consanguineous parents compared to non-consanguineous parents.

Moreover, the distribution of infant *VAX1* rs4752028 genotypes in cases and in controls according to consanguinity is presented in Appendix A32. It showed that out of the total sample with VAX1 rs4752028 genotype recorded (120 CL/P and 35 CP infant genotypes compared to 188 controls), there were 118 CL/P and 35 CP compared to 167 controls that had been genotyped and their parental consanguinity status recorded. The oral cleft phenotype of two of the NSOFC cases was not reported. There were no statistically significant differences between the three *VAX1* rs4752028 genotypes in cases (P= 0.123) or in controls (P= 0.19). In addition, Appendix A33 shows the distribution of infant *VAX1* rs4752028 genotypes in NSOFC cases compared to controls according to parental consanguinity. No significant differences were found (P> 0.05).

Table 5.59 show the distribution of infant VAX1 rs4752028 alleles in NSOFC consanguineous parents compared to controls. More NSOFC including CL/P and CP cases with consanguineous parents were found with the rare C allele compared to controls. However the difference was not significant for CP. For NSOFC, P< 0.001, OR: 3 and 95% CI (1.55 to 5.4); for CL/P, P= 0.001, OR: 2.97 and 95% CI (1.54 to 5.76); and for CP, P= 0.059, OR: 2.44 and 95% CI (0.97 to 6.16).

Table 5.59: Distribution of infant *VAX1* rs4752028 alleles in cases and controls with consanguineous parents.

Allele type	NSOFC (%)	CL/P (%)	CP (%)	Control (%)
T*	135 (78.5)	101 (77.7)	34 (81.4)	166 (91.2)
C	37 (21.5)	29 (22.3)	8 (18.6)	16 (8.8)
Total	172 (100)	130 (100)	42 (100)	182 (100)
P-value	<0.001**	0.001**	0.059	
OR (95% CI)	3 (1.55-5.4)	2.97 (1.54-5.76)	2.44 (0.97-6.16)	

X^2=28.28, df=2, P<0.001**

* Common allele

**Significant level at P≤ 0.05

5.5.7.2 Infant *VAX1* rs7078160

The prevalence of the rare homozygous genotype (AA) according to parental consanguinity is presented in Appendix A34. This genotype was found to be more frequent in infants with consanguineous parents (61.5% compared to 38.5%) but when the correlation was corrected for column comparison, this difference was not statistically significant (P> 0.05).

Table 5.60 shows the distribution of infant *VAX1* rs7078160 genotypes in cases and in controls according to consanguinity. Out of the total sample with *VAX1* rs7078160 genotype

record (157 NSOFC; 122 CL/P and 35 CP infant genotype compared to 186 controls), there was 149 NSOFC (116 CL/P and 33 CP) compared to 164 controls that had been both genotyped and their parental consanguinity recorded.

After Bonferroni correction for column comparison for NSOFC cases, there was a significantly higher prevalence of the homozygous common allele genotype (GG) in infants with consanguineous parents compared to the prevalence of the heterozygous genotype (AG) ($P<0.05$). Appendix A35 showed no statistically significant differences between NSOFC cases and controls infant *VAX1* rs7078160 genotypes.

The prevalence of the rare (A) allele in NSOFC compared to controls in infants with consanguineous parents is presented in Appendix A36. Although the rare allele prevalence was greater in NSOFC (11%) than in controls (7.7%), and the chi square and P value was statistically significant (X^2=6.11, df=2, P= 0.047) when comparing CL/P, CP and controls, the differences between each phenotype (CL/P and CP compared to controls) was not statistically significant ($P>0.05$).

Table 5.60: Distribution of infant VAX1 rs7078160 genotypes in cases and controls according to parental consanguinity. (d) Four undiagnosed NSOFC cases

Consanguinity	**NSOFC**			**CL/P**			**CP**			**Control**		
	GG*	AA	AG	GG*	AA	AG	GG*	AA	AG	GG*	AA	AG
Yes (%)	73 (62)	6 (54.5)	7 (33.3)	53 (60.9)	6 (54.4)	6 (33.3)	20 (66.7)	0	1 (33.3)	79 (56.4)	2 (100)	10 (45.5)
No (%)	44 (38)	5 (45.5)	14 (66.7)	34 (39.1)	5 (45.5)	12 (66.7)	10 (33.3)	0	2 (66.7)	61 (43.6)	0	12 (54.5)
Total (%)	117 (100)	11 (100)	21 (100)	87 (100)	11 (100)	18 (100)	30 (100)	0	3 (100)	140 (100)	2 (100)	22 (100)
Total infants	149[C,d]			116[C]			33[C]			164[C]		
X^2(df), P-value	6.05 (2), 0.048**			4.62 (2), 0.099			1.31 (1), 0.252			2.55 (2), 0.279		

* Homozygous common allele genotype is the reference

**Significant level P≤ 0.05

[C] Out of the157 NSOFC (122 CL/P and 35 CP) 186 controls, 8 NSOFC (6 CL/P, two CP), and 22 controls, did not have their genotype and/or their paternal consanguinity information completed.

5.5.8 Haplotype-Based Association Test (HBAT)

The haplotype-based association test (HBAT) was carried out on the five SNPs included in this study (rs7078160, rs4752028, rs2235371, rs2013162 and rs2235375) and showed that two significant haplotype blocks were associated with NSOFC (Table 5.61). The first significant block (GCCCC (# 3)) included the common alleles of four SNPs and the rare allele of *VAX1* rs4752028 (P= 0.021). The second significant block (GTCAC (#6)) included the common four SNPs and the rare allele of allele of *IRF6,* rs2013162 (P= 0.01)

Table 5.61: Haplotypes showing transmission distortion.

Number	Haplotype	Estimation of frequency	Z	P-value
1	GTCCC	0.446	0.928	0.353
2	GTCAG	0.212	-1.045	0.295
3	GCCCC	0.086	2.299	0.021*
4	GTCCG	0.076	1.744	0.081
5	ACCCC	0.051	-0.929	0.977
6	GTCAC	0.037	-2.578	0.01**
7	GCCAG	0.026	-0.879	0.379
8	ACCAG	0.018	0.007	0.995

**Significant level P≤ 0.05

5.6 Gene-environmental interaction (GIE)

Maternal gene-environmental interactions (GEI) for *IRF6* (rs2013162, rs2235375 and rs2235371) and *VAX1* (rs4752028 and rs7078160) were examined using two study designs: case-only and case-control study designs. The case-only study design includes assessment of GEI for maternal gene variants and alleles. We selected maternal gene for GEI analysis according to the literature (shi et al., 2008) and the log-linear model on the interaction between maternal genetic variants and infant genetic variants. Although the sample size is not adequate, the preliminary results of log-linear model showed a significant interaction between maternal gene and infant gene (P<0.05). This analysis result is presented in Appendix (A37).

5.6.1 Maternal *IRF6* rs2013162 gene-environmental interaction

The interactions between maternal *IRF6* rs2013162 variants and environmental factors were analysed using two approaches: case-only and case-control approaches.

5.6.1.1 Case-only study design approach

The maternal *IRF6* rs2013162 rare homozygous allele genotype (AA) and heterozygous genotype (CA) were compared to the common homozygous allele genotype (CC) in NSOFC cases with regard to different environmental factors (Appendix A38). Mothers with homozygous rare allele genotype (AA) who were using antipyretic medication in the 1st trimester period (P= 0.012, OR: 9 and 95% CI: 1.62 to 49.91), folic acid supplement in the pregestation period (P= 0.047, OR: 8.22 and 95% CI: 1.03 to 65.72), experiencing abdominal pain in the 1st trimester period (P= 0.013, OR: 6.57 and 95% CI: 1.49 to 29.01) and depression in the 1st trimester (P = 0.048, OR: 8.11 and 95% CI: 1.01–64.84) are more likely to have a NSOFC offspring. Mothers with heterozygous allele genotype (CA) who were exposed to fever during the three-month pregestation period (OR: 0.26 and 95% CI: 0.08 to 0.84) and X-ray in the 1st trimester period (P<0.001, OR: 0.09 and 95% CI: 0.03 to 0.34) were significantly less likely to have NSOFC infants. Mothers with heterozygous allele genotype (CA) who were using folic acid supplement in the pregestation period were significantly more likely to have NSOFC infants (P= 0.014, OR: 6.83 and 95% CI: 1.47 to 31.66).

The maternal *IRF6* rs2013162 rare A allele was compared to the common C allele in NSOFC cases in relation to different environmental factors (Appendix A39). Mothers with rare A allele who were using antipyretic medication in the 1st trimester period (P= 0.025, OR: 2.34

and 95% CI: 1.12 to 4.9) and folic acid supplement during the pregestation period (P= 0.012, OR: 2.57 and 95% CI: 1.23 to 5.37) were significantly more likely to have NSOFC infants. Mothers with rare A allele who were exposed to fever during the three-month pregestation period (P= 0.019, OR: 0.28 and 95% CI: 0.09 to 0.81), X-ray in the 1st trimester period (P < 0.001, OR: 0.19 and 95% CI: 0.07 to 0.5), drank tap water as their main drinking water source compared to bottled water (P= 0.043, OR: 0.51, 95% CI: 0.26 to 0.98) and ingested iron supplement in the 1st trimester period (P= 0.04, OR: 0.57 and 95% CI: 0.33 to 0.97) were significantly less likely to have NSOFC infants.

5.6.1.2 Case-control study design approach.

Appendix A40 shows the distribution of the maternal *IRF6* rs2013162 genotypes in NSOFC cases compared to controls according to different environmental factors including maternal illness, medication, stress, supplements, paternal smoking and maternal domestic chemical exposure.

5.6.1.2.1 IRF6 rs2013162 maternal homozygous rare allele genotype (AA):

Statistically significant differences between cases and controls were found for the interaction between the homozygous rare allele genotype (AA) and environmental factors: maternal folic acid supplementation was significantly less utilized by NSOFC mothers in the 1st

trimester period (P= 0.003, OR: 0.1 and 95% CI: 0.02 to 0.50) and significantly more utilized by NSOFC mothers in the pre-gestation period compared to controls (P= 0.023); maternal multivitamin supplementation in the pre-gestation period was significantly more utilized by NSOFC mothers compared to controls (P= 0.023); paternal Jorak waterpipe smoking occurred significantly more often in NSOFC families compared to controls (P= 0.005); and maternal passive smoking was significantly more likely to be identified in NSOFC mothers exposed to secondary smoking compared to controls (P= 0.006, OR: 10.42 and 95% CI: 1.61 to 67.34). Maternal stress was reported significantly more often in NSOFC mothers compared to controls: P= 0.003, OR: 10.06 and 95% CI: 1.98 to 51.04 for mothers complaining of family problems; P= 0.015, OR: 6.13 and 95% CI: 1.33 to 28.21 for mothers complaining of being under stress; P= 0.023 for maternal depression in the 1st trimester and P= 0.007, OR: 14.86 and 95% CI: 1.42 to 154.99 for maternal abdominal pain in the 1st trimester. Maternal exposure to chemicals in pre-gestation and the 1st trimester period was significantly more likely in NSOFC mothers compared to controls (P= 0.012, OR: 6.9 and 95% CI: 1.4 to 33.92).

5.6.1.2.2 IRF6 rs2013162 maternal homozygous common allele genotype (CC):

There were statistically significant differences between cases and controls for the maternal homozygous common allele genotype (CC) frequency in: maternal exposure to antibiotics in the pre-gestation period, with significantly more NSOFC mothers reporting antibiotics ingestion compared to controls (P= 0.003, OR: 5.8 and 95% CI: 1.58 to 21.24); pre-gestation maternal illness with significantly more NSOFC mothers complaining of illness in the pre-gestation period compared to controls (P= 0.01, OR: 2.73, and 95% CI: 1.25 to 5.97); pre-gestation maternal common cold/ flu with significantly more infection reported by NSOFC mothers compared to controls (P< 0.001, OR: 10.06 and 95% CI: 2.85 to 35.48); pre-gestation maternal fever (P= 0.009, OR: 4.3 and 95% CI: 1.34 to 13.82) and paternal

waterpipe smoking (P= 0.022, OR: 4.26 and 95% CI: 1.13 to 16.1), also significantly more reported by NSOFC mothers compared to controls; and there were significant differences in maternal drinking water types between tap water and Zamzam water (P= 0.024).

5.6.1.2.3 IRF6 rs2013162 maternal heterozygous genotype (CA):

The heterozygous genotype (CA) of *IRF6* rs2013162 was significantly higher in cases compared to controls in: mothers exposed to illness in the pre-gestation period (P= 0.004, OR: 4.2 and 95% CI:1.48 to 11.92); mothers exposed to common cold/flu in the pre-gestation period (P= 0.032, OR: 5.37 and 95% CI: 1.44 to 19.98); family problems (P= 0.034, OR:2.24 and 95% CI: 1.05 to 4.74); maternal complaints of being under stress (P= 0.36, OR: 2.14 and 95% CI: 1.04 to 4.39); and family history for birth defects (P= 0.037, OR:2.78 and 95% CI: 1.31 to 5.86). The heterozygous genotype (CA) was significantly higher in controls than cases with maternal exposure to incense in the pre-gestation and 1st trimester periods (P= 0.029, OR: 0.46, CI: 0.23 to 0.93) and (P= 0.028, OR: 0.46 and 95% CI: 0.23 to 0.92) respectively.

5.6.1.2.4 Chi square correction using Bonferroni correlation:

Chi square correlations using Bonferroni corrections for the three maternal *IRF6* rs2013162 genotypes in cases compared to controls according to different environmental factors showed a statistically significant case and control difference in the maternal homozygous rare allele genotype (AA) frequency for: mothers using folic acid supplementation, with significantly more control mothers ingested folic acid supplementation in the 1st trimester compared to NSOFC mothers; maternal passive smoking, which was significantly elevated in NSOFC mothers exposed to secondary smoking compared to controls; maternal stress, was reported significantly more often in NSOFC mothers compared to controls (including mothers

complaining of family problems, being under stress, and abdominal pain in the 1st trimester); maternal exposure to chemicals in the pre-gestation and 1st trimester period, occurring statistical significantly more often in NSOFC mothers compared to controls. There was a significantly higher frequency of the common maternal homozygous genotype (CC) in cases compared to controls in groups with: maternal exposure to antibiotics in the pre-gestation period; pre-gestation maternal illness; pre-gestation maternal common cold/ flu; and pre-gestation maternal fever. The heterozygous genotype (CA) was significantly more likely to be observed in cases compared to controls in: mothers exposed to illness in the pre-gestation period, mothers exposed to common cold/flu in the pre-gestation period; mothers experiencing family problems; mothers complaining of being under stress; and family history for birth defects. The heterozygous genotype (CA) was significantly higher in controls than in cases for mothers exposed to incense in the pre-gestation and 1st trimester periods.

5.6.2 Maternal *IRF6* rs2235375 GEI

The maternal *IRF6* rs2235375 genotypes in NSOFC cases were compared to each other to determine the influence of different environmental factors in a case-only study design and were compared to controls.

5.6.2.1 Case-only study design approach

The maternal *IRF6* rs2235375 rare homozygous allele genotype (GG) and heterozygous genotype (CG) were compared to the common homozygous allele genotype (CC) in NSOFC cases to determine the influence of different environmental factors (Appendix A41).

Mothers with rare homozygous allele genotype (AA) who were using folic acid supplement during the pregestation period (P= 0.045, OR: 0.23 and 95% CI: 0.05 to 0.97) and complaining of being under stress (P= 0.016, OR: 0.3 and 95% CI: 0.1 to 0.8) were significantly less likely to have NSOFC infants.

The relationship between different environmental factors and the maternal *IRF6* rs2235375 rare G allele was compared to the common C allele in NSOFC cases (Appendix A42).

Mothers with rare G allele who had consanguineous marriages (P= 0.042, OR: 1.6 and 95% CI: 1.02 to 2.52) were significantly more likely to have NSOFC infants. Mothers with rare G allele who were complaining of being under stress (P= 0.01, OR: 0.52 and 95% CI: 0.32 to 0.84) and were exposed to X-ray during the 1st trimester period (P= 0.02, OR: 0.21 and 95% CI: 0.05 to 0.87) were significantly less likely to have infants with NSOFC

5.6.2.2 case-control study design approach.

Appendix A43 shows the distribution of maternal *IRF6* rs2235375 genotypes for NSOFC cases compared to controls according to environmental factors including maternal illness, medication, stress, supplements, paternal smoking and maternal domestic exposure.

5.6.2.2.1 IRF6 rs2235375 maternal homozygous rare allele genotype (GG):

There were significantly more NSOFC cases with the maternal homozygous *IRF6* rs2235375 rare allele genotype (GG) compared to controls for: maternal common cold/flu in the pre-gestation period (P< 0.001, OR: 12.08 and 95% CI: 2.66 to 54.89); maternal fever in the pre-gestation period (P= 0.007, OR:6.73 and 95% CI: 1.42 to 31.94); maternal high blood pressure in the 1st trimester period (P= 0.028); paternal waterpipe smoking (P= 0.018, OR: 4.48 and 95% CI:1.18 to 17.07) and paternal Jorak smoking (P= 0.013). There was a

statistically significant difference between cases and controls for the maternal rare allele genotype (GG) in maternal drinking water between tap water and bottle waters (P= 0.033).

5.6.2.2.2 IRF6 rs2235375 maternal common allele genotype (CC):

There were significantly more NSOFC cases with the maternal *IRF6* rs2235375 common allele genotype (CC) compared to controls in: maternal illness in the pre-gestation (P= 0.005, OR:7.92and 95% CI: 1.6 to39.29) and 1st trimester periods (P= 0.013, OR:3.56 and 95% CI: 1.27 to 9.92); maternal common cold/flu in the pre-gestation period (P= 0.013, OR: 10.11 and 95% CI:1.18 to 86.98); folic acid supplementation in the pre-gestation period (P= 0.026, OR: 0.12 and 95% CI: 0.04 to 0.31); paternal waterpipe (P= 0.041 OR:4.93 and 95% CI: 0.95 to 25.58) including Jorak smoking (P= 0.026); and maternal stress including mothers experiencing family problems (P= 0.001, OR: 5.67 and 95% CI:1.89 to 16.99)and mothers complaining of being under stress (P= 0.014, OR: 4.22 and 95% CI: 1.57 to 11.36). There was a statistically significant difference between cases and controls for the maternal common allele genotype (CC) in maternal drinking water types (Zamzam compared to tap water P= 0.027, OR16 and 95% CI: 1.32 to 194.63).

5.6.2.2.3 IRF6 rs2235375 maternal heterozygous genotype (CG):

There were statistically significant differences between cases and controls for the maternal heterozygous *IRF6* rs2235375 genotype (CG) in: maternal illness in the pre-gestation period (P= 0.009, OR: 3.59 and 95% CI: 1.31 to 9.78); folic acid supplementation in the 1st trimester ((P= 0.02); mother complaining of being under stress (P= 0.019); mothers complaining of family problems (P= 0.036, OR: 2.32 and 95% CI: 1.05 to 5.14); and family history of birth defects (P= 0.027, OR: 2.62 and 95% CI:1.22 to 5.62).

5.6.2.2.4 Chi square correlation using Bonferroni correction:

Chi square correlation using Bonferroni correction for maternal *IRF6* rs2235375 genotypes in cases compared to controls according to different environmental factors showed a significantly higher rate of possessing the maternal homozygous rare allele genotype (GG) in cases compared to controls in maternal flu/common cold infection in the pre-gestation period; and in fever in the pre-gestation period. There were significantly higher maternal homozygous common allele genotype (CC) frequencies in cases compared to controls in: maternal illness in the pre-gestation and 1st trimester periods; flu/common cold infection in the pre-gestation period; fever in the pre-gestation period; maternal folic acid supplementation; and maternal stress (mothers complaining of being under stress and having family problems). There were significantly higher findings of the maternal heterozygous genotype (CG) in cases compared to controls for: maternal illness in the pre-gestation period; lack of maternal folic acid supplementation; maternal stress (mothers complaining of family problems and being under stress); and family history of birth defects.

5.6.3 Maternal *IRF6* rs2235371

Maternal *IRF6* rs2235371 genotypes in NSOFC cases could not be compared to each other in relation to the different environmental factors as no mothers had a homozygous rare allele genotype (TT).

Appendix A44 shows the distribution of NSOFC maternal *IRF6* rs2235371 genotypes for NSOFC cases compared to controls according to environmental factors including maternal illness, medication, stress, supplements, paternal smoking and maternal domestic exposure. There were significantly more NSOFC cases with the maternal homozygous common allele genotype (CC) compared to controls for: maternal antibiotics ingestion in the pre-gestation

period (P= 0.004); maternal anti-emetic medication ingestion in the 1st trimester period (P= 0.027); maternal illness in the pre-gestation and 1st trimester periods (P< 0.001 and P= 0.011, respectively); common cold/flu infection in the pre-gestation and 1st trimester periods (P< 0.001 and P= 0.032 respectively); fever in the pre-gestation period (P= 0.011); paternal Jorak smoking (P= 0.002), maternal stress exposure (mothers complaining of family problems (P= 0.003) and stress (P= 0.003)); and family history of birth defects (P= 0.014). There were significantly more controls with the common maternal homozygous allele genotype (CC) compared to NSOFC cases for: maternal folic acid supplementation in the 1st trimester period; maternal calcium supplementation in the 1st trimester period (P= 0.031); incense exposure in the pre-gestation and 1st trimester periods (P= 0.026 and P= 0.02, respectively); and mothers drinking Zamzam water (P= 0.015).

All variables shown to be significant in 5.86 were proven to be significant after Chi square correlation using Bonferroni correction for the three maternal *IRF6* rs2235371 genotypes in cases compared to controls stratified according to different environmental factors.

5.6.4 Maternal *VAX1* rs4752028 GEI

The maternal *VAX1* rs4752028 genotypes in NSOFC cases were compared to each other with regard to different environmental factors and were compared to controls.

5.6.4.1 Case-only study design approach

The maternal *VAX1* rs4752028 rare homozygous allele genotype (CC) and heterozygous genotype (CT) were compared to the common homozygous allele genotype (TT) in NSOFC cases with regard to different environmental factors (Appendix A45).

Mothers with rare homozygous allele genotype (CC) who were exposed to high blood pressure during the 1st trimester period were significantly more likely to have NSOFC infant (P= 0.022, OR: 11.22 and 95 % CI: 1.41 to 89.4)

The maternal *VAX1* rs4752028 rare C allele was compared to the common T allele in NSOFC cases with regard to different environmental factors (Appendix A46). Mothers with a rare C allele genotype who were exposed to fever during the 1st trimester period (P<0.001, OR: 0.11 and 95% CI: 0.05 to 0.26) and experiencing depression in the 1st trimester (P= 0.04) were significantly less likely to have NSOFC infants

5.6.4.2 Case-control study design approach

Appendix A47 shows the distribution of maternal *VAX1* rs4752028 genotypes for NSOFC cases compared to controls according to environmental factors including: maternal illness, medication, stress, supplements, paternal smoking and maternal domestic exposure.

5.6.4.2.1 VAX1 rs4752028 maternal homozygous rare allele genotype (CC):

There were significantly fewer NSOFC cases with the maternal homozygous rare allele genotype (CC) compared to controls in: maternal asthma in the pre-gestation period (P= 0.047); maternal iron supplementation in the pre-gestation period (P= 0.047) and maternal exposure to incense (0.024).

5.6.4.2.2 VAX1 rs4752028 maternal homozygous common allele genotype (TT):

There were significantly more maternal homozygous *VAX1* rs4752028 common allele genotypes (TT) in NSOFC cases compared to controls in: maternal antibiotics ingestion in the pre-gestation period (P= 0.004, OR: 3.6 and 95% CI:1.42 to 9.1); maternal anti-emetic medication ingestion in the 1st trimester period (P= 0.018, OR:2.49 and 95% CI: 1.15 to 5.39); maternal illness in the pre-gestation and 1st trimester periods (P< 0.001, OR: 4.48 and

95% CI:2.11 to 9.51 and P= 0.01, OR: 2.03 and 95% CI: 1.18 to 3.5 respectively); maternal common cold/flu infection in the pre-gestation period (P< 0.001, OR: 7.02 and 95%: 2.55 to 19.32); maternal fever in the pre-gestation period (P= 0.03, OR: 2.79 and 95% CI: 1.07 to 7.25); multivitamin supplementation ingestion in the pre-gestation period (P= 0.044, OR:5.12 and 95% CI: 1.04 to 25.19); paternal Jorak smoking (P= 0.024, OR: 5.23 and 95% CI:1.06 to 25.73); mothers complaining of being under stress and suffering from maternal depression in the 1st trimester period (P= 0.05, OR: 1.7 and 95% CI: 1 to 2.9 and P= 0.027, OR: 5.1 and 95% CI: 1.04 to 25.1, respectively); and family history of birth defects (P= 0.005, OR: 2.13 and 95% CI: 1.24 to 3.64). There were significantly more maternal homozygous common allele genotypes (TT) in controls compared to cases for maternal folic acid supplementation in the 1st trimester period, (P= 0.023, OR: 0.54 and 95% CI: 0.32 to 0.92) and type of maternal drinking water (P= 0.001).

5.6.4.2.3 VAX1 rs4752028 maternal heterozygous genotype (CT):

There was a statistically significant difference between cases and controls for the maternal heterozygous genotype (CT) in maternal calcium supplementation in the 1st trimester period (P= 0.025, OR: 0.17 and 95% CI: 0.033 to 0.93) and in paternal waterpipe smoking (P= 0.018).

5.6.4.2.4 Chi square correlation using Bonferroni correction:

Chi square correlation using Bonferroni correction for the three maternal *VAX1* rs4752028 genotypes in NSOFC cases compared to controls according to different environmental factors found a significantly higher frequencies of maternal homozygous common allele genotypes (TT) in NSOFC cases compared to controls in: maternal antibiotics ingestion in the pre-gestation period; maternal anti-emetic medication ingestion in the 1st trimester period; maternal illness in the pre-gestation and 1st trimester periods; maternal common cold/flu infection in the pre-gestation period; maternal fever in the pre-gestation period;

paternal Jorak smoking; and maternal stress and depression in the 1st trimester period. There was significantly more maternal homozygous common allele genotypes (TT) in controls compared with cases in maternal folic acid supplementation in the 1st trimester period, maternal multivitamin supplementation in the pre-gestation period, and in mothers drinking Zamzam water. There was a significantly higher level of maternal heterozygous genotypes (CT) in NSOFC controls compared to cases in maternal calcium supplementation in the 1st trimester period.

5.6.5 Maternal *VAX1* rs7078160 GEI

The relationships between maternal *VAX1* rs7078160 genotypes in NSOFC cases were compared to each other and to controls.

5.6.5.1 Case-only study design approach

The maternal *VAX1* rs7078160 rare homozygous allele genotype (AA) and heterozygous genotype (AG) were compared to the common homozygous allele genotype (GG) in NSOFC cases with regard to different environmental factors (Appendix A48).

Mothers with a homozygous rare allele genotype (AA) who were exposed to high blood pressure (P= 0.011, OR: 15 and CI: 1.86 to 120.86), had infants' fathers using a waterpipe (P= 0.016, OR: 5.54 and 95% CI: 1.38 to 22.23) were significantly more likely to have NSOFC infants. In contrast, they were less likely to have NSOFC infant when exposed to chemicals during the pregestation period (P< 0.001, OR: 0.06, CI: 0.01 to 0.24) if they had homozygous rare AA allele genotype.

Mothers with heterozygous allele genotype (AG) who were exposed to incense during the 1st trimester period (P= 0.008, OR: 8.15 and 95% CI: 1.71 to 38.87) and ingested iron supplements during the pregestation period (P= 0.011, OR: 4.83 and 95% CI: 1.44 to 16.27) were significantly more likely to have NSOFC infants. However, they were less likely to have NSOFC infant with parental consanguinity when having a heterozygous allele AG genotype (P= 0.04, OR: 0.42 and 95% CI: 0.18 to 0.96).

The maternal *VAX1* rs7078160rare allele (A) was compared to the common allele (G) in NSOFC cases with regard to different environmental factors (Appendix A49).

Mothers with rare A allele that were exposed to high blood pressure during the 1st trimester (P = 0.01, OR: 5.87 and 95% CI: 1.42 to 24.3) and fathers were using waterpipe smoking device (P = 0.01, OR: 2.67 and 95% CI: 1.28 to 5.56) were significantly more likely to have NSOFC infants. Mothers with rare A allele who were exposed to chemicals during the pregestation period (P< 0.001, OR: 0.19 and 95% CI: 0. 11 to 0.34) were significantly less likely to have NSOFC infants.

5.6.5.2 Case-control study design approach

Appendix A50 shows the distribution of maternal *VAX1* rs7078160 genotypes among NSOFC cases compared to controls with regard to various environmental factors, including maternal illness, medication, stress, supplements, paternal smoking and maternal domestic exposure.

5.6.5.2.1 VAX1 *rs7078160 maternal homozygous rare allele genotype (AA)*

There were statistically significant differences between cases and controls for GEI between the maternal homozygous rare allele (AA) and maternal smoking (P= 0.032).

5.6.5.2.2 **VAX1** *rs7078160 maternal homozygous common allele genotype (GG)*

There was a statistically significant increase in NSOFC cases compared to controls for GEI between maternal homozygous common allele genotype (GG) and maternal antibiotic ingestion during the pre-gestation period (P= 0.013, OR: 2.81 and 95% CI: 1.21 to 6.5), maternal anti-emetic medication ingestion during the 1st trimester period (P= 0.041, OR: 2.14 and 95% CI: 1.02 to 4.5); maternal illness in the pre-gestation and 1st trimester periods (P< 0.001, OR: 3.54 and 95% CI: 1.75 to 7.13; and P= 0.029, OR: 1.77 and 95% CI: 1.06 to 2.96, respectively); maternal common cold/flu infection during the pre-gestation period (P< 0.001, OR: 5.39 and 95% CI: 2.11 to 13.77); paternal Jorak smoking (P= 0.01, OR: 6.15 and 95% CI: 1.3 to 29); family problems and mothers complaining of being under stress (P = 0.025, OR: 1.82 and 95% CI: 1.08 to 3.09; and P= 0.033, OR: 1.7 and 95% CI: 1.04 to 2.84) respectively); and family history of congenital anomalies (P=33, OR: 0.01.73 and 95% CI: 1.04 to 2.89). There were significantly more controls compared to NSOFC cases with the maternal common allele genotype (GG) in maternal folic acid supplementation during the 1st trimester (P= 0.028, OR: 0.57 and 95% CI: 0.35 to 0.94) and in mothers drinking Zamzam water compared to tap water (P= 0.005, OR: 8.92 and 95% CI: 1.92 to 41.52).

5.6.5.2.3 Chi square correction using Bonferroni correlation

Chi square correlation using Bonferroni correction for the three infant *VAX1* rs7078160 genotypes in NSOFC cases compared to controls according to different environmental factors revealed a significantly larger number of NSOFC cases with the maternal homozygous rare allele genotype (AA) compared to controls with parental consanguinity. Moreover, there was a significantly larger number of NSOFC cases with the maternal homozygous common allele genotype (GG) compared to controls in maternal antibiotics

ingestion in the pre-gestation period; maternal anti-emetic medication ingestion during the 1st trimester period; maternal illness during the pre-gestation period; maternal common cold/flu infection during the pre-gestation period; mothers complaining of being under stress; family problems; and family history of congenital anomalies. There were significantly more controls compared to NSOFC cases for GEI between maternal homozygous common allele genotype (GG) and maternal folic acid supplementation during the 1st trimester and mothers drinking Zamzam water.

5.6.6 Multi-nominal logistic regression analysis for case-only study design gene-environmental interaction

Multi-nominal logistic regression analysis was carried out to identify significant gene-environmental interactions among NSOFC cases. The homozygous common allele genotype for each included SNP was set as a reference for analysis (see Table 5.62). For maternal rs2013162, the homozygous rare allele genotype (AA) was significantly related to antipyretic medication in the 1st trimester period (P= 0.027, OR: 10.18 and 95% CI: 1.31 to 79.1) and abdominal pain in the 1st trimester period (P= 0.031, OR: 7.4 and 95% CI: 1.2 to 45.51) among NSOFC cases. The heterozygous allele genotype (AC) was significantly related to folic acid pre-gestation (P= 0.0167, OR: 6.78 and 95% CI: 1.41 to 33.49) and fever pre-gestation (P= 0.025, OR: 0.23 and 95% CI: 0.06 to 0.83) among NSOFC cases.

For maternal *VAX1* rs7078160, the homozygous rare allele genotype was significantly related to paternal waterpipe (P= 0.013, OR: 6.95 and 95% CI: 1.5 to 32.2) and maternal exposure to high blood pressure during the 1st trimester period (P= 0.05, OR: 11.2 and 95% CI: 1 to 125.73) among NSOFC.

There was no significant gene-environmental interaction as shown by regression analysis for maternal *IRF6* rs2235375 and maternal *VAX1* rs4752028.

Table 5.62: Multi-nominal logistic regression analysis for case-only study design gene-environmental interaction including significant factors.

SNP	P value OR 95% (CI)	
Maternal rs2013162[b]		
Variables	**AA**	**CA**
Antipyretic medication 1st trimester	0.027** 10.18 (1.31-79.1)	0.132 2.74 (0.74 -10.13)
Folic acid pre-gestation	0.351 3.83 (0.23-64.42)	0.017** 6.87 (1.41-33.49)
Maternal exposure to X-ray 1st trimester	a	0.947 1.07 (0.16-7.01)
Fever pre-gestation	a	0.025** 0.23 (0.06-0.83)
Depression in the 1st trimester	0.274 6.22 (0.24-164.39)	0.56 1.96 (0.2-18.98)
Abdominal pain 1st trimester	0.031** 7.4 (1.2-45.51)	0.61 1.36 (0.42-4.38)
Maternal *IRF6* rs2235375[b]		
Variables	**GG**	**CG**
Folic acid pre-gestation	0.051 0.23 (0.05-1)	0.385 0.59 (0.18-1.94)
Mother complains of being under stress	0.508 0.75 (0.32-1.75)	0.76 0.88 (0.38-2.03)
Maternal *VAX1* rs4752028 [C]		
Variables	**CC**	**CT**
Exposure to incense 1st trimester	0.51 0.63 (0.16-2.51)	0.811 0.92 (0.45-1.88)
Fever 1st trimester	0.48 0.46 (0.06-3.85)	0.14 0.42 (0.14-1.33)

Maternal VAX1 rs7078160[d]		
Variables	**AA**	**AG**
Iron supplementation pregestation	a	0.017 5.18 (1.34-20.11)
High blood pressure 1st trimester	0.05** 11.2 (1-125.73)	a
Paternal waterpipe	0.013** 6.95 (1.5-32.2)	0.622 0.69 (0.16-2.95)
Exposure to chemicals pregestation	0.909 0.9 (0.15-5.55)	0.362 1.56 (0.6-4.02)
Exposure to incense 1st trimester	0.346 0.42 (0.07-2.54)	0.8 0.88 (0.34-2.31)
Consanguinity	0.662 1.41 (0.3-6.7)	0.073 0.45 (0.19-1.08))

** Significant value P≤ 0.05

a. No value either because the parameter is set to zero or the value is redundant

b. The reference category is the common homozygous allele genotype CC.

[C]. The reference category is the common homozygous allele genotype TT

d. The reference category is the common homozygous allele genotype GG

Chapter 6: Discussion

6.1 Introduction

This thesis is the first report of a national multicentre based study that measured the prevalence and investigated the aetiology of NSOFC in Saudi Arabia. Knowing that the prevalence and aetiology of NSOFC varies geographically and in different ethnic groups, it is important that this is investigated in every population (Mossey and Little, 2002). Although the ethnicity of the Middle East is considered Caucasian or 'white', defined as a person having origins in any of the original peoples of Europe, the Middle East, or North Africa (Risch et al., 2002; Lewonin 2005), geographically, the Middle East is located between the three main continents (Asia, Africa and Europe) which makes it unique in in its genetic admixture of the three populations.

Saudi Arabia is the largest country in the Middle East with a population of 29 million and estimated 300,000 births per year. It consists of three main regions: Central, Western and Eastern. The Western region, which encompasses one third of the Saudi population, is further divided into Makkah and Maddina regions. The Central region also encompasses one third of the Saudi population, and consists mainly of the Riyadh region (Ministry of Health, 2010).

6.1.1 Barriers and facilitators in carrying out this research:

This retrospective case triad/control triad study on NSOFC in Saudi Arabia faced some methodological difficulties and barriers throughout its fieldwork, during data collection and genotyping. There were also other aspects that went well. These difficulties and facilitators are described in the following points.

6.1.1.1 Ethical approval:

The sample design in our research was aiming to include referral centres from each region covering most of the NSOFC cases in the three included cities. This was achieved in Jeddah and Maddina. However, in Riyadh, King Faisal Specialised Hospital and Research Center which is a cleft centre in Riyadh had an ongoing research project that is already underway on oral cleft patients. As they did not want to exhaust their patients, approval to carry out our research was not gained in their hospital. Therefore, we recruited our sample from four hospitals in Riyadh.

Moreover, the request for research and ethical approval were preformed multiple times with a structured proposal form unique for almost each hospital (nine requests for the 11 included hospitals). In addition, some of the ethical requests took almost a year to receive an approval by some hospitals. It would be useful if a more standardized procedure could be used for obtaining ethical permission across Saudi Arabia; and perhaps other parts of the Middle East also, especially for rare diseases and low exposures research.

In Jeddah, funding support was received from King Abulaziz University Research Centre in King Abulaziz University, which also provided the genetic lab (Princess Al-Jawhara Albrahim Center of Excellence in Research of Hereditary Disorders) and technicians. In addition, the Ministry of Health supported the research by facilitating the ethical approval in their hospitals.

Moreover, support was received from the staff of the hospitals by directing us during the paper work and helping us to find a Principle Investigator (PI working in the same hospital was one of the requirement for research approval).

6.1.1.2 Identification of cases and ascertainment

Although nurses and doctors were very supportive and tried to facilitate the researcher reaching NSOFC patients, sub-mucosal cleft palate and bifid uvula are known to be difficult to identify clinically (Gosain et al., 1996). Therefore, it was excluded from the investigation.

In addition, as our research was carried out in three cities at the same time, with long destination (almost 10 hours by car) between cities, one research coordinator was not enough, Therefore, more than one research coordinators were assigned for collecting the saliva and filling the questionnaire. However, efforts were made to limit their number and improve the research ascertainment as was discussed in the material and method section (4.2.3.4).

6.1.1.3 Sample collection

Once nurses and doctors identified a NSOFC case, they facilitated parental contact with the researcher. Nevertheless, some of the parents were reluctant to provide saliva sample or allow us to take saliva from their infants. Consequently, the sample size for assessing environmental risk factors that needed only a questionnaire exceeded that assessing the genetic risk factors. In addition, fathers were more reluctant than mothers in providing their saliva (16 fathers out of 189 infant-parental triad controls did not give their saliva).

The sample was transported from Riyadh and Maddina to the genetic lab in Jeddah through a very hot atmosphere that reaches over 40 degrees. Thus it needed transportation on wet ice, hand-to-hand, to ensure sample protection.

6.1.1.4 Questionnaire responses

Most of the parents agreed to answer the questionnaire. Even though, we faced some difficulties in getting the general characteristics of the infant (neonatal head circumferential and infant neonatal length) when the infant was not born in the same hospital. As the neonatal head circumferential and infant neonatal length are not always written on the infant's vaccination card.

Also, there are some points that need to be considered in measuring SES level for Saudis. Parental income, by some, was considered "personal information". However, this problem was managed by asking about their occupation. Occupation type could give an estimated value for the salary. In addition, measuring the household income is different from other populations. The majority of the Saudi population families are financially supported by the fathers, even if the mother is working. Still, as most of the mothers are not working (86% of our sample), the household income could be considered equal to paternal income.

For questions related to maternal medication ingestion (yes/no questions) in the 1st trimester, mothers answered directly and clearly. But, they did not always remember their names or/and their doses.

Further, there was a risk of bias in questions related to smoking. The social acceptance of smoking, especially for woman, was expected to lead to underestimation of this risk factor. However, as this problem is in both cases and controls, it should not have had a significant effect on our results. Still, we tried to overcome this problem by asking more questions related to; smoking type and severity.

Passive smoking intensity was reported in another research to be difficult to measure (Sabbagh et al., published/ in press 2015). Still, as most of the mothers in cases and controls

were not working (87% of mothers in cases and controls), exposures to 2nd hand smoking were easier to measure as it is mainly in houses from other family members.

Moreover, stress level is difficult to measure. The differences in abilities to cope with stress; the secrecy of private life when it comes to family problems; and the differences in abilities to describe stress, make stress a challenging area to study. However, these difficulties were found in both cases and controls. In addition, measures were taken through; asking questions lightly in comfortable tone; and assuring confidentiality of the information as much as possible, in order to gain a correct answer.

On the other hand, information related to parental consanguinity was easy to get and directly answered by the parents.

Finally, parents answered most of the question willingly. However, these points mentioned above are important to consider in future research carried out in Saudi Arabia, in order to reduce the risk of bias.

6.1.1.5 Genotyping

Genotyping was carried out in Saudi Arabia according to the Saudi Government rules. The genetic lab in Princess Al-Jawhara Albrahim Center of Excellence in Research of Hereditary Disorders accepted to carry out the research in their lab, providing the technician and most of the hardware equipment required for genotyping. However, enzymes were bought abroad and transferred through the Saudi border. This process needed the Saudi FDA approval which took longer time than expected resulting in ruining the enzymes. Therefore, materials were requested and bought multiple times. These problems prolonged the research laboratory procedure timeline.

6.1.1.6 Saudi Arabia as a place for research

Saudi Arabia is a good environment for research in many aspects. The government of Saudi Arabia supports research. Funds are provided through research centres. Labs and technicians with good experience are available. Governmental hospitals assess the researcher in providing and facilitating sample recruitment.

In addition, Saudi Arabia's unique population characteristics such as; their high birth rate and high consanguinity prevalence, makes it a good candidate for parental consanguinity, hereditary disease and birth defect research. In addition, Saudis, especially in the Western Region, have been of mixed ethnicity for hundreds of years. People from all over the world, of different ethnic origins, have travelled to Makkah and Maddina on pilgrimage, then, settled and mixed races through marriage. Additionally, Saudi Arabia has a unique geographic location between the three continents; Asia, Africa and Europe. As a result of this, it can be difficult to group people according to their ethnicity in Saudi Arabia, although they are, generally considered Caucasian (Risch et al., 2002; Lewonin 2005).

Moreover, as mothers in Saudi Arabia are mainly housewives, maternal domestic exposures could be easier to measure than other parts of the world were women are exposed to both occupational and household exposures.

Finally, it is recommended that the awareness is roused amongst the Saudi population about the importance of research. This would help to improve the population support and research approval.

6.2 Part I: The prevalence of NSOFC in Saudi Arabia

The birth prevalence of NSOFC in the three main cities in Saudi Arabia over the two-year period was 1.17/1000 live-births, which was lower than the global birth prevalence (1.25/1000) for NSOFC (Mossey and Modell, 2012a) and also lower than the mean overall prevalence of NSOFC for all studies carried out in Saudi Arabia and in neighbouring Middle Eastern countries (1.25 per 1000 live-births) (Sabbagh et al., 2012). Only two studies have previously reported the prevalence of NSOFC in Saudi Arabia. These studies were based from single hospitals, conducted in 1991 and in 1993 and carried out in Riyadh and in Alqaseem (Borkar et al., 1993; Kumar et al., 1991). The Borkar et al. (1993) study reported a lower incidence of 0.3/1000 births in Riyadh, whereas the Kumar et al. (1991) study reported a much higher incidence of 2.19/1000 births in Al-Quasseem as compared to the global prevalence of NSOFC.

6.2.1 Comparisons between the prevalence of NSOFC in different cities (table 5.1)

In Jeddah city, the prevalence of NSOFC (0.8/1000 births) was lower than in Riyadh (1.07/1000 births). However, the highest NSOFC prevalence was for Maddina city (1.88/1000 births); this prevalence was also higher than the global figure. It is notable that there is a higher prevalence of consanguinity reported in Maddina (67.2%) compared to Jeddah (44%) and Riyadh (60%) (El-Hazmi et al., 1995; El Mouzan et al., 2008). The differences in prevalence between cities might also indicate different aetiological risk factors for each region that would need further investigation by studies with larger sample sizes to elucidate accurately.

6.2.2 Prevalence of NSOFC phenotypes and description of its sub-phenotypes (table 5.2)

The prevalences of CL/P (0.89/ 1000 live births) and CP (0.28/ 1000 live births) were lower than the global prevalences (0.94 and 0.31 /1000 live births, respectively). However, the prevalences of CP in Riyadh (0.4/ 1000 live births) and in Maddina (0.36/ 1000 live births) were higher than both the Jeddah CP prevalence (0.13/1000 live births) and the global figure (0.31/ live 1000) (Mossey and Modell, 2012a). This could be explained by the high prevalence of consanguinity for both Riyadh and Maddina compared to Jeddah, as consanguinity was reported in previous studies to be associated with CP more than with CL/P (Sabbagh et al., 2013). The relationship between consanguinity and NSOFC will be discussed in more detail in the following section.

The finding from our study that the birth prevalence of CL (0.47/1000) is higher than that of CLP (0.42/1000) differs from the global finding and those of previous studies, which reported a higher birth prevalence of CLP (from 40 to 45%) compared to CL (20 to 25%) (Mossey and Modell, 2012a). Our findings, especially in Maddina (where the CL prevalence is 0.85 compared to the CLP prevalence of 0.67 /1000 births and which also has a higher NSOFC prevalence), do not support Mossey and Modell (2012a), who suggested that decreases in the ratio between CLP and CL are found in regions with a low prevalence of NSOFC. They also suggested that a trend for less severe NSOFC would be associated with low prevalence of NSOFC, which is different from what we found in this study as the prevalence of bilateral CL/P in Jeddah, which has low NSOFC prevalence, was 48.4% (15 cases out of the total 31 CL/P cases), higher than the prevalence of bilateral CL/P (20.9%; 9 cases out of the total 43 CL/P cases) in Maddina which has the highest NSOFC prevalence. These differences were significant (P= 0.02). These findings and the differences in the

prevalence of NSOFC sub-phenotypes could indicate the involvement of different aetiological factors and may highlight a specific trend of NSOFC phenotypes in the respective cities.

Our study showed that the prevalence of NSOFC (133 cases) and CL/P (101 cases) in males ((82 cases (61.7%) and 66 cases (65.3%) respectively) was higher than in females (51 cases (38.3%) and 35 cases (34.7%), respectively). The severity of CL/P is related to this, with an increase in the male to female ratio reaching almost 4:1 in complete bilateral CLP. This finding was also reported by Mossey and Modell (2012), who conveyed a male to female ratio of about 2:1 and that the M:F ratio increased with the severity of CL/P.

The prevalence of CP was higher in females (10 (62.5%)) compared to males (6 (37.5) in Jeddah and Maddina. These findings are similar to the global figures (Mossey and Modell, 2012a). Riyadh, on the other hand, had a higher number of males (10 cases) compared to females (6 cases) in the CP group. Only one other study in the Arabian Middle Eastern countries has reported a higher prevalence of CP in males (Aqrabawi, 2008). A local aetiological factor or poor ascertainment of CP could lie behind the increase in CP ratio in males during the two year period that was included in this research. Additional studies are needed to further clarify the relationship between various risk factors and the manifestation of CP, and how this might differ between genders.

For OFC sub-phenotypes, our study found that left sided unilateral CL (29 cases (21.7%)) and CLP (16 (12.5%)) was more common than the right sided CL/P (13 cases (9.7%)), similar to global figures (Mossey and Modell, 2012a; Rakotoarison et al., 2012; Rajab and Thomas, 2001; Rajabian and Sherkat, 2000).

6.2.3 Prevalence of consanguinity in NSOFC cases (table 5.3)

Consanguineous relationships have been suggested to increase the prevalence of congenital anomalies especially in recessive gene disorders (Pritchard and Korf, 2008). Saudi Arabia has a high rate of consanguineous marriages although this rate varies across regions (El-Hazmi et al., 1995).

The prevalence of consanguinity in NSOFC cases reported by this study was 81 cases (65.9%), which is higher than the prevalence reported by Al-Johar et al. (2008) (54.4%) and the prevalence reported by both El Mouzan et al. (2008) and EL-Hazmi et al. (1995) for the Saudi community (57% and 57.7%, respectively) (El-Hazmi et al., 1995Al-Johar et al., 2008; El Mouzan et al., 2008;). In addition, the prevalence of consanguinity in NSOFC infants in Maddina (77.4%), in Riyadh (56.1%) and in Jeddah (58.6%) were almost similar or slightly higher than the prevalence of consanguinity in the general population as reported by El Mouzan et al. (2008), which were 67.2%, 60% and 44% for each city respectively. Of the total infants with consanguineous parents, 44 cases (54.3%) were first cousins, which was also almost similar to the El-Hazmi et al. (1995) findings (41%) for the general population of the Saudi community. The higher prevalence of consanguineous marriages in NSOFC cases compared to the general population could indicate that consanguinity is a risk factor in the aetiology of NSOFC; a theory supported by previous research (Stoll et al., 1991; Reddy et al., 2010; Sabbagh et al., 2014). However, in order to confirm this relationship, a case-control design study is needed. Therefore, the role of consanguinity will be re-examined in part II of this thesis.

Looking at the different NSOFC phenotypes, the prevalence of CP cases with consanguineous parents was more than that of CL/P. Although this difference was not

statistically significant (P= 0.11), it is supported by other studies that found a higher prevalence of CP infants related to consanguineous parents (Ravichandran et al., 2012; IPDTOC Working Group, 2011). This relationship could explain the higher prevalence of CP in Maddina compared to Jeddah, as Maddina has a higher prevalence of consanguinity. Furthermore, our study found no relationship between consanguinity and gender in NSOFC (P= 0.559).

The prevalence of CL was also higher in infants with consanguineous parents (63.3%) than those with non-consanguineous parents (36.7%). Although this relationship was not statistically significant, it could explain why the prevalence of CL was higher than CLP in the Saudi population. In addition, the relationship between consanguinity and CL could explain the higher prevalence of CL compared to CLP in Maddina (0.89 compared to 0.67/1000 births) as compared to Jeddah (0.35 compared to 0.33/1000 births). Studies in countries with low consanguinity demonstrated a higher prevalence of CLP compared to CL (Bonaiti et al., 1982; Zlotogora, 1997; Cooper et al., 2006), supporting these models.

Furthermore, 70.2% of complete clefting of the lip and 66.7% of bilateral clefting of the lip occurred in infants with consanguineous parents. This could suggest that consanguinity could be related to the pattern and severity of NSOFC phenotypes rather than the prevalence. More details on the relationship between consanguinity and NSOFC identified from previous research is discussed in the paper "Parental consanguinity and non-syndromic orofacial clefts in children; a systematic review and meta-analyses" (Sabbagh et al., 2014) presented in Appendix B3**.**

6.3 Part II: Aetiology of NSOFC

The aetiology of NSOFC is multifactorial and includes three groups of risk factors; environmental risk factors, genetic risk factors and an interaction between the gene and environmental risk factors.

6.3.1 Environmental risk factors

A case-control study was designed to investigate the environmental risk factors for NSOFC. This type of study plays an important role in disease prevention strategies (Mossey and Castilla, 2001). The most important environmental factors that were described to be associated with NSOFC in previous research include: maternal exposure to antibiotics, disease, stress, smoking and chemicals, and these factors were investigated in this project.

A questionnaire was used to retrospectively record exposure to environmental factors. This method of collecting data could be subjected to recall bias which results from parents of cases over reporting exposures compared to controls. However, previous studies that assessed recall bias in case-control studies measuring the influence of exposures on birth defects, reported minimal or irrelevant bias effect. They compared the use of affected controls with unaffected controls and reported minimal changes in OR between both control groups. Accordingly, they concluded satisfactory results in using unaffected controls (Khoury et al., 1994; Swan et al., 1992).

To determine the sample size required for our study to yield statistically significant results, we measured the sample size that was expected to be obtainable in two years (as mentioned in the material and methods section (Paragraph 4.2.2)) and we also included referred cases

that were 18 months or less in age that attended the targeted hospitals to increase the sample size. Two other methods were carried out to further define the sample size: a systematic review of the prevalence of clefting in Saudi Arabia (Sabbagh et al., 2012); and estimation of the prevalence of NSOFC from exposure to the risk factors identified from previous studies. Accordingly, to reach 80% sample power, we need at least 105 cases compared to 105 controls. Our sample exceeded this number reaching 205 cases and 244 controls.

The proportion of CP (25.8%) in the study sample is similar to the global proportion, ranging from 20-25% (Mossey and Modell, 2012). The overall proportions of CL (38%) in the included sample were slightly higher than CLP (36.8%) which differs from the global figures which show a higher proportion of CLP (40-45%) compared to CL (20 to 25%) (Mossey and Modell, 2012); however, these values from this part of the study were similar and supported to our findings that was discussed in the prevalence part 1 section of this study.

The frequency prenatal diagnosis of CLP (12.6%) was more common than for CL (8%), which is supported by the results of Stoll et al. (2000). However, CL (8%) diagnosis frequency was higher than that reported by Stoll et al. (2000) (3.7% for isolated CL) and did not agree with their report stating that there were more diagnosed cases with associated anomalies compared to isolated anomalies (15.6% for isolated CL/P compared to 5% for CL/P with associated anomalies).

However, similar findings regarding the prevalence of associated anomalies was reported by Stoll et al., (2000), and CP appeared to be more closely associated with other anomalies compared with other oral cleft phenotypes (Stoll et al., 2000). This could be explained by Mossey and Modell, (2012) who suggested the possibility of other birth anomalies

stimulating detailed examination, leading to detecting mild CP, which could be overlooked if it occurred alone. Besides, Rittler et al. 2011 reported that 7.1% of NSOFC cases diagnosed as isolated cleft at birth were found to be associated with other birth anomalies after one year. Additional details regarding the prevalence of associated anomalies are described in Appendix B (B1, B4 and B5).

6.3.1.1 Parental age and infant characteristics (table 5.5 and 5.6):

This study found no association between NSOFC and parental age, in concordance with findings of previous studies (Jamilian, 2007; Kanaan, 2008; Al-Sahafi, 2010). In 2012, a meta-analysis carried out to assess the relationship between parental age and NSOFC found heterogeneity between studies looking at parental age and their relationship to CL/P. However, a relationship was found between parental age and CP only (Herkrath et al., 2012).

Neonatal weight was similar in both groups (cases and controls) in our study, in line with previous findings (Leitte and Koifman, 2009; Welch and Hunter, 1980), although in their epidemiological study in France, Bonaiti et al. (1982) reported lower birth weights for infants with CP.

Congenital anomalies have been reported to be higher in twins compared to singletons (Glinianaia et al., 2008). Although the number of twins in this study sample was low (12 pairs of infants) and they were not monozygotic, there were a significantly higher number of twins in the study group (11 pairs of infants) compared to controls (one pair) ($P= 0.002$). Twinning was associated with CL/P ($P= 0.019$ for CL and $P< 0.001$ for CLP) but not with CP ($P= 0.24$). Previous research did not support this relationship (Kot and Kruk-Jeromin, 2005; Nordström et al., 1996). However, these were not case-control study and they included

both mono and dizygotic twins. In 2012, a Danish national research study found no increased risk of NSOFC in twins compared to singletons although they reported that the recurrence risk for clefts was greater in twins than in non-twin siblings and that the heritability estimate was over 90% which indicates a genetic factor effect (Grosen et al., 2012). Finally, the relationship found in this study with dizygotic twins suggested an environmental risk factor rather than a genetic risk factor. In addition, the relationship with twins being significant with CL/P cases rather than CP could suggest a mechanical interference risk factor interrupting the development of the craniofacial structure. Further investigation to clarify the relationship is suggested.

6.3.1.2 Socio-economic status (SES) (table 5.7):

It has been suggested that SES was associated with NSOFC. This was explained by the adverse effect of low SES on parental healthcare and lifestyle (Taghavi et al., 2012). However, our study did not support this relationship. Our finding could be related to the generally higher SES of Saudis compared to other communities (Trading economics; http://www.tradingeconomics.com/saudi-arabia/gdp), the availability of free governmental healthcare and education. Also medication and supplementations are provided free in the Ministry of Health and governmental hospitals (Saudi Ministry of Economy and Planning statistical book, 2013).

The number of CP cases identified in our study from rural areas was significantly greater than urban areas as compared to controls. This finding is supported by another study (Al-Sahafi, 2010). This could be a valid relationship, or it could be an artefact, because families with NSOFC infants tend to seek treatment in cities, whereas unaffected infants from rural areas tend to have vaccinations locally, potentially skewing ascertainment of the 'matched' control group for residency comparison. Nevertheless, our study found no significant

difference in the prevalence of CL/P between rural and urban areas which is similar to Stoll et al. (1991) in north-eastern France. Future study that includes new-born cases and controls from rural and urban areas is recommended for confirming their relationship to CP.

6.3.1.3 Pregnancy planning and the effect of sibling order (table 5.8 and 5.9):

Recently, Martelli et al. (2010) carried out a case-control study in Minas Gervais to evaluate environmental risk factors for CL/P in 100 children. They reported no relationship between CL/P and paternal age, pregnancy order or inter-pregnancy interval. Although our study did not find a relationship between NSOFC phenotypes and sibling order ($P>0.05$), a significant relationship between CL and inter-pregnancy interval duration was anticipated ($P= 0.01$). Further investigation followed by a systematic review and meta-analysis is recommended to clarify this relationship.

Our study reported more mothers planning their pregnancy in the control group (45.4%) compared to CLP (40.5%) and CP (40%). Although this finding was not significant ($P>0.05$), it is supported by Mossey et al. (2007) who reported a statistically significant relationship between pregnancy planning and NSOFC based on a case-control study. However, they also found a greater difference between cases and controls if the mother was a smoker, which is an aetiological factor (maternal smoking) rarely reported in our sample.

6.3.1.4 Family history and consanguinity (table 5.10 to table 5.13):

Family history of NSOFC was reported to increase the chance and recurrence of NSOFC (Krapels, 2005; Jia et al., 2011; Zandi and Heidari, 2011). In our study the prevalence of NSOFC with a family history of birth defects was 41.2% (84 cases), lower than what was reported by Aljohar et al. (2008) (54.4%) but similar to the findings of Borkar et al. (1993) (42%). Another study in Riyadh, Saudi Arabia reported a very low number of patients with

family history of congenital anomalies (8%) (Kumar et al., 1991). In our study, the difference between cases and controls in the prevalence of reported family history for all types of birth defects was statistically significant only for CLP infants (P= 0.012). However, the difference between cases and controls in the prevalence of reported family history of OFC was significantly higher in cases compared to controls for all types of NSOFC (P< 0.001) with the highest prevalence of family history for CLP (39.2%). This was similar to findings from Jia et al. (2011), who reported a significant difference between cases and controls (P= 0.001) and a higher prevalence of family history for CL/P (13.6%) compared to CP (4.5%).

Prevalence of consanguinity in the case-control section of our study (Part II) (56.7%) was lower than the prevalence section (Part I) (65.5%). However, it was similar to the prevalence of the control group (59.2%). In addition, the prevalence of CP cases with consanguineous parents was higher (65.4%) than controls which agrees with results presented in Part I. A role for consanguinity in OFC is also supported by previous research some of which, although not all, found a significant relationship between consanguinity and NSOFC. Grouping these studies in meta-analysis resulted in the identification of a significant relationship (OR: 1.83 and 95% CI: 1.31 to 2.54). Moreover, consanguinity in this meta-analysis was also reported to be more related to CP (OR: 1.89 and 95% CI: 1.14 to 3.31) compared to CL/P (OR: 1.56 and 95% CI: 1.18 to 2.07) (Sabbagh et al., 2014) (Appendix B3). However, in order to confirm parental consanguinity relationship with NSOFC, it is necessary to match cases with controls in residency or take controls from rural areas, as they are more likely to be involved in consanguineous marriages.

The most prevalent type of consanguineous marriage in our study group was between first cousins (60.5%), as compared to other types of consanguineous marriages and compared to

controls (58.9%) but there was no significant difference between these (P= 0.643). Similar findings were reported by the Sabbagh et al. (2014) meta-analysis which identified a significant association between first cousin consanguineous marriages and NSOFC (OR: 1.49 and 95% CI: 1.07 to 2.07) (Appendix B3).

6.3.1.5 Maternal supplementation (table 5.14 and 5.15):

Maternal supplement ingestion during the first trimester showed a significant decrease in the chance of having a child with NSOFC for both folic acid and calcium supplementation. Folic acid supplementation was associated with decreased risk of NSOFC (P= 0.009, OR: 0.59 and 95% CI: 0.39 to 0.88) and of CLP (P< 0.001, OR: 0.34 and 95% CI: 0.2 to 0.58). This association was in accordance with a meta-analysis carried out by Bodovinac et al. (2007) that included five prospective studies, from which a significant relationship was identified between folic acid and NSOFC occurrence (OR: 0.55 and 95% CI: 0.32 to 0.95). This association was statistically significant for CL/P (OR: 0.51 and 95% CI: 0.32 to 0.95) but not for CP (P= 0.18, OR: 1.19 and 95% CI: 0.43 to 3.28) (Badovinac et al., 2007). On the other hand, although not significant, our study found a tendency of increased risk of CL in mothers using folic acid in the pregestation period (P= 0.22, OR: 1.62 and 95% CI: 0.75 to 3.5). This finding is supported by a recent population base case-control study in Northern Netherlands that investigated the relationship between peri-conceptional folic acid supplementation and the different oral cleft phenotypes. They reported "duration of exposure-response effect" for folic acid maternal ingestion that increased the risk of cleft lip when ingested in the pregestation period (Rozendaal et al., 2013).

Although many studies have reported a significant association between CL/P and maternal multivitaminsingestion, others found no significant relationship (Goh et al., 2006). This supports our findings of a non-significant association betweenmultivitamins and CL/P (P=

0.38, OR: 0.74 and 95% CI: 0.38 to 1.45 for CL; and P= 0.31, OR: 0.7 and 95% CI: 0 .3 to 1.4 for CLP).

For calcium supplementation, a statistically significant decreased risk of having an infant with NSOFC was observed (P= 0.014, OR: 0.37 and 95% CI: 0.16 to 0.84).

Although calcium supplementation was more often utilized by the mothers in the control group compared to the mothers in the three NSOFC phenotype groups (CL, CLP and CP), CP was the only phenotype that showed a statistically significant difference with supplementation compared to the control group (P= 0.024). This finding was different from that reported by Jia et al. (2011), who found a statistically significant decrease chance of having an infant with CL/P after maternal calcium supplementation (P= 0.02, OR: 0.66 and 95% CI: 0.47 to 0.93) but not for CP (P= 0.33, OR: 0.81 and 95% CI: 0.52 to 1.24). The significant relationship for calcium supplementation and CP in the Saudi population could be related to the severe levels of Vitamin D deficiency in Saudi women who have one of the highest Vitamin D deficiency levels in the world (Elshafie et al., 2012). Vitamin D is known to play an important role in the maintenance of serum (ionised) calcium levels (Nordin, 2010).

Finally, Public Health Organizations in planning their policies for preventing oral clefts should call for further research that confirms the relationship between folic acid and NSOFC. The differences in reports suggesting; decrease in CL/P if folic acid is ingested in the 1st trimester; increase of CL if folic acid is ingested in the pregestation period; and, the presence of mandatory folate fortification of flour in Saudi Arabia (Saudi Standard of fortification, 2001) makes it a challenging intervention.

6.2.1.6 Maternal illnesses (table 5.16 to 5.19):

Maternal illness and maternal common-cold/flu infection in the three months pre-gestation were significantly reported more frequently by CL/P and CP mothers (for maternal illness: P= 0.024, OR: 2.04 and 95% CI: 1.1 to 3.82 for CL; P< 0.001, OR: 3.69 and 95% CI: 2.04 to 6.68 for CLP; and P= 0.013, OR: 2.39, 95% CI: 1.19 to 4.81 for CP; and for common-cold/flu: CL, P= 0.014, OR: 2.45, 95% CI: 1.23 to 6.28; CLP, P< 0.001, OR: 6.34 and 95% CI: 3.11 to 13.18; and CP, P= 0.031, OR: 2.66 and 95% CI: 1.06 to 6.63) compared to controls. For the 1st trimester, maternal illness was more frequent in CL/P and CP mothers compared to control. However, it was only significant for CL (P= 0.037, OR: 1.74 and 95% CI: 1.04 to 2.94). For maternal common-cold/flu in the 1st trimester, the statistical significant relationship was only for CP (P= 0.037, OR: 2.03 and 95% CI: 1.03 to 3.98). This could be because mothers might not be aware of their pregnancies until the end of their second gestation month, which is a critical time for craniofacial development. Furthermore, the three-month pre-gestation period covers the time around conception, which could affect embryonic development (Mossey and Castilla, 2001). The positive relationship between maternal illness and having a child with NSOFC has been reported by other studies as well (Czeizel, 2002; Krapels, 2005; Edwards, 2006; Hashmi et al., 2010). Recently, Luteijn et al., (2014) carried out a meta-analysis to assess the relationship between maternal influenza infection in the 1st trimester and birth defects. They reported a significant relationship with NSOFC (OR: 1.96 and 95% CI: 1.33 to 2.91) including CL (OR: 3.12 and 95% CI: 2.20 to 4.42).

The adverse outcome of maternal illness and common-cold/flu infection could be the result of an associated adverse effect that accompanies the illness such as hyperthermia, stress or

from the medication ingested to manage these illnesses. Therefore, we will further discuss maternal fever and antipyretics in the coming paragraphs to clarify this relationship.

Our study also found a significant relationship between maternal diabetes and CP (P= 0.018). This finding is supported by that of Carinci et al. (2005), who reported a significant association between familial diabetes and CP only (P = 0.001) in Southern Italy. Another case-control population-based study in the US reported a significant relationship with CL/P (Spilson et al., 2001). It is noteworthy, however that the prevalence of diabetes among pregnant women in the control group was lower than what was reported in previous studies carried out in a one hospital set in Saudi Arabia which ranged from 8 to 12% (Khwaja et al., 1989; Ardawi et al., 2000; Al-Hakeem, 2006).

Other diseases were rarely reported by mothers, suggesting a larger sample size is needed to measure their exposure effects.

6.3.1.7 Maternal hyperthermia (fever) and antipyretics (table 5.20 and 5.29):

Researchers have reported an association between hyperthermia and many adverse outcomes ranging from abortion to birth defects depending on the development stage, duration of hyperthermia and the severity of fever (Graham and Edwards, 1998; Walsh et al., 1998; Edwards, 2006). This could be explained by the effects of hyperthermia that can cause the death of proliferating cells, interruption of the normal sequence of developmental gene activities, or damage to the embryonic vascular system during a critical stage of development (Rockett et al., 2001; Omori et al., 2014).

In our study hyperthermia was associated with an increased risk of NSOFC in general and of CLP (P= 0.048, OR: 1.98 and 95% CI: 1.01 to 3.63 for NSOFC and P= 0.008, OR: 2.78 and

95% CI: 1.28 to 6.05 for CLP). In addition, we observed that a higher number of mothers in the control group ingested antipyretics (12.3%) compared to the NSOFC group (6.3%) including all its phenotypes. However, a statistically significant difference was only observed in the total NSOFC cases compared to controls in the pregestation period (P= 0.03, OR: 0.48 and 95% CI: 0.24 to 0.94). The idea of decreasing the chance of having an infant with NSOFC by maternal antipyretic medication ingestion is also supported by results from Hashmi et al. (2010). The effect of antipyretic medications could explain the relationship between maternal illness and NSOFC, suggesting that the disease symptoms might be the underlying reason behind development of NSOFC. To understand the effect of hyperthermia directly, future research is needed that recognizes the source of hyperthermia, along with the severity, duration and cause. Also, studies assessing the relationship between hyperthermia and cleft phenotype severity are recommended.

6.3.1.8 Other medications (table 5.21 to 23):

The main medications that were reported to be ingested by mothers were antibiotics. Maternal ingestion of antibiotics was found to increase the risk of NSOFC and CL/P (NSOFC: P= 0.003, OR: 2.81 and 95% 1.38 to 5.72 for the pre-gestation period and P= 0.023, OR: 1.95 and 95% CI: 1.09 to 3.51 for the 1st trimester period; and CL/P: P= 0.012, OR: 2.96 and 95% CI: 1.22 to 7.16 for CL; and P= 0.03, OR: 2.64 and 95% CI: 1.07 to 6.55 for CLP for the pregestation period). Although there were more mothers reporting ingesting antibiotics in CP cases compared to controls, the difference was not statistically significant (P= 0.08). Similar findings were reported by Lin et al. (2012) who found significantly increased CL/P risk (OR: 2.0 and 95% CI: 1.0 to 4.1) among mothers using Amoxicillin in

pregestation period. They also found that the relationship was not significant for CP (OR: 1.0 and 95% CI: 0.4 to 2.3).

Moreover, a nationwide cohort study of 806,011 live births in Denmark concluded that although maternal antibiotic ingestion was not a major risk factor for NSOFC, there was a significant relationship between certain classifications of antibiotics (tetracycline, sulfamethizole, doxycycline, trimethoprim) and NSOFC (Mølgaard-Nielsen and Hviid, 2012). These types of antibiotics and amoxicillin were reported to cross the placenta (Nathanson et al., 2000). However, the exact mechanism of its effect on cleft lip and palatal development remains unknown. One explanation suggested is that the increase in NSOFC incidence is due to the underlying diseases treated with these antibiotic rather than the antibiotic itself. In our study there was a significant relationship between maternal diseases in the pregestation period and NSOFC. However, Lin et al. (2012) reported that after adjusting the OR for maternal infection, a relationship still exist between CL/P and amoxicillin. Besides, trimethoprim was reported to be a dihydrofolate reductase inhibitor that may counteract the effect of folic acid in pregnant women (Zimmerman et al., 1997). Our study was not able to stratify the sample according to maternal antibiotic types because mothers in both cases and controls were not able to remember the name of their medication. Therefore, future research that assesses the amount, type, duration and dosage of antibiotics is still needed. The mechanism of action for these antibiotics on infant development also needs to be clarified in upcoming research.

6.3.1.9 Maternal stress (table 5.24 to 5.26):

Maternal stress was considered one of the risk factors for NSOFC and was investigated through measuring the effect of stressful events (Carmichael et al., 2007). In this study,

maternal stress was measured through recording information on maternal experience of: general stress, family problems, depression, severe morning sickness, threatened abortion and abdominal pain.

Maternal stress was found to significantly increase the risk of NSOFC and its different phenotypes to almost double the baseline chance (maternal experience of stress: P< 0.001, OR: 2.1 and 95% CI: 1.43 to 3.11 for NSOFC; P= 0.04, OR: 1.74 and 95% CI: 1.01 to 3 for CL; P= 0.001, OR: 2.53 and 95 % CI: 1.48 to 4.32 for CLP; and P= 0.009, OR: 2.53 and 95% CI: 1.2 to 4.09 for CP). Family problems during the pre-gestation and 1st trimester period were significantly over-reported by mothers in the study group: P= 0.001, OR: 1.97 and 95% CI: 1.31 to 2.95 for NSOFC; P= 0.04, OR: 1.79 and 95% CI: 1.03 to 3.13 for CL; P= 0.004, OR: 2.21 and 95% CI: 1.28 to 3.84 for CLP; and P= 0.04, OR: 1.96 and 95% CI: 1.04 to 3.67 for CP. A relationship between stress and NSOFC has been supported by many studies (Saxen, 1974; Czeizel and Nagy, 1986; Laumon et al., 1996; Carmichael and Shaw, 2000; Radojicic et al., 2007). The effect of maternal stress resulting from stressful events could be related to elevated maternal corticotrophin-releasing hormones and corticosteroid levels (Hobel et al., 1999; Angelica Montenegro et al., 1995), a hypothesis reinforced by findings that corticosteroid medication itself could increase the risk of NSOFC (Carmichael et al., 2007a). Another explanation by which stress might cause birth defects is from the negative behaviours that might result from or cope with stress such as reduced nutrient intake or smoking (Carmichael et al., 2007b).

Our study has identified more mothers experiencing severe morning sickness or using anti-emetic medication in the NSOFC group compared to controls. Anti-emetic medications were reported to be consumed by 12.1% of NSOFC, 14.5% CL and 6.6% of controls, a statistically significant difference with P= 0.043 and 0.019 has been identified respectively.

It has been suggested that the occurrence of severe morning sickness or ingestion of anti-emetic medication predicts a less favourable pregnancy outcome. This model is supported by Miller (2002), who suggested that severe morning sickness can lead to loss of about 5% of original maternal weight, which could result in birth defects (Miller, 2002). Moreover, Jia et al. (2011) found a reduced chance of having an infant with NSOFC after maternal weight gain during pregnancy (OR: 0.15, 95% CI: 0.034–0.63).

Zhang and Cai (1991) carried out a cross-sectional study on 1867 women to assess possible causes for maternal severe vomiting during pregnancy. They reported a twofold increase in the prevalence of severe maternal vomiting associated with paternal smoking. Moreover, an association between severe maternal smoking and foetal growth retardation was reported (OR: 1.4, 95% CI: 0.9 to 2.3) (Zhang and Cai, 1991). Accordingly, the relationship found between severe morning sickness and NSOFC could be related to maternal passive smoking and paternal smoking. More studies that stratify samples according to paternal smoking and maternal passive smoking are indicated to clarify this relationship.

On the other hand, other studies suggested a reduced chance of having an infant with NSOFC or no relationship with maternal nausea and vomiting (Czeizel et al., 1984; Czeizel et al., 2002; Badovinac et al., 2007; Molina-Solana et al., 2013). One explanation for these findings was that woman experiencing nausea and vomiting tend to stop their consumption of alcohol; however, this is not the situation for Saudi woman who do not drink alcohol because of their Islamic beliefs. Another explanation suggested for the effect of nausea reported by some studies was because of the effects of hormonal and high oestrogen levels in early pregnancy and/ or dietary changes, although this possibility still needs further clarification (Hook, 1976; Mossey, 2001a).

6.3.1.10 Smoking (table 5.27 to 5.34)

A significant relationship between maternal smoking and having an infant with NSOFC has been reported by multiple studies (Little et al., 2004; Jia et al., 2011; Zhang et al., 2011). In this study, the number of maternal smokers was quite low, making it impractical to analyse this risk factor. Bassiony (2009) also reported a low prevalence of smoking in females (9%) compared to males (22.6%) in Saudi Arabia. However, smoking statistics may be underestimated because of tradition and social stigma associated with smoking, particularly for females (Bassiony, 2009). In the current study, the prevalence of paternal smoking (36%) was higher than that reported by Bassiony (2009), but similar to the findings of Fida and Abdelmoneim (2013).

Paternal smoking was suggested to play a role in the aetiology of NSOFC either directly by affecting sperm development and increasing the frequency of abnormal sperm or indirectly through environmental contamination with tobacco, increasing maternal exposure to smoke (Weisberg, 1985; Zhang et al., 1992; De and Aitken, 2000; Berthiller and Sasco, 2000).

In general, we found no significant relationship between paternal smoking and NSOFC, although a significant relationship was reported by previous studies (Little et al., 2004a; Jianyan et al., 2010). However, in these studies, the intensity and the types of paternal smoking was not assessed. Our study showed that paternal smoking of 20 or more cigarettes/day was associated with a higher risk of having a child with NSOFC (P= 0.013, OR: 3.07 and 95% CI: 1.25 to 7.55) and CP (P = 0.012, OR: 5.23, 95 % CI 1.33 to 20.58) compared to controls, indicating a dose-response relationship. This finding is supported by the results of Shaw et al. (1996), who also reported a dose-response relationship between paternal smoking and NSOFC for fathers smoking 20 cigarettes or more. In addition, our study examined the type of smoking device and found a significant relationship between

paternal waterpipe smoking and CL/P (for CL: in the pre-gestation period P= 0.04, OR: 2.45 and 95% CI: 1.04 to 5.77, and in the 1st trimester period P= 0.046, OR: 2.56 and 95% CI: 1 to 6.62; for CLP: in the pre-gestation period P< 0.001, OR: 3.83 and 95% CI: 1.73 to 8.48, and in the 1st trimester period P= 0.001, OR: 4.11 and 95% CI: 1.73 to 9.77). Other previous studies found that waterpipe smokers have significantly higher carboxyhaemoglobin concentrations than cigaratte smokers as well as higher carbon monoxide levels, and they have higher serum cotinine levels because of the larger amount of tobacco consumed during waterpipe smoking compared with cigarette smoking (Zahran et al., 1985; Ardawi et al., 2007). Also, waterpipe contains heavy metals such as arsenic, cobalt, chromium, and lead (Shihadeh, 2003). The knowledge of the effect of waterpipe and its content on infants' development in the literature is still in its preliminary stage. However, waterpipe smoking component that are similar to other tobacco smoking devices were described by The Centers for Disease Control Prevention and Surgeon General in their report (2010) to have adverse effect on spermatogenesis and infant development. For instance, carbon monoxide is thought to have a direct effect on spermatogenisis through oxidative DNA damage (Fraga et al., 1996; Shen et al., 1997). In addition, it has an indirect effect through maternal second hand smoking causing fetal hypoxia that lead to fetal growth retardation (Li et al., 2004). Also, nicotine was suggested to play a role in inducing tobacco birth defects and still birth when combined with other smoking component (Gupta and Subramoney, 2006). The possibility of waterpipe component causing second hand smoking has not been yet investigated. Nevertheless, waterpipe is expected to produce different teratogenic effect than other devices. This is because the structure of the device and the waterpipe size has an effect on the amount of tobacco consumption. In addition, the heating temperature of the waterpipe device (450C) is less than other devices (half that of cigarette smoking) (Maziak et al., 2004).

In their review of waterpipe smoking, Maziak et al. (2004) addressed waterpipe smoking as an important epidemic global concern and identified a lack of knowledge and awareness concerning dealing with waterpipe smoking and its effects. In addition, they recommended the need to assess differences and similarities among the different waterpipe types (Maziak et al., 2004). Following their recommendations, we sub-grouped the paternal waterpipe smoking into Jorak and Moasel smokers. Both types of waterpipe smoking are smoked by the Saudi population; Jorak is an older, traditional form, which is typically smoked by older people, while Moasel is a newer form that is smoked by younger generations, and contains different fruit incenses (Shihadeh, 2003). We found that Jorak smoking was associated with statistically significant increased risks of having an infant with NSOFC, including CL/P, if smoking occurred in the three months prior to pregnancy (P= 0.001, OR: 6.34 and 95% CI: 1.8 to 22.23 for NSOFC; P= 0.01, OR: 5.58 and 95% CI: 1.3 to 23.95 for CL; and P< 0.001, OR: 9.62 and 95% CI: 2.48 to 37.27 for CLP). For the first trimester period, paternal Jorak smoking was also associated with significant increased NSOFC risk: P= 0.01, OR: 4.66 and 95% CI: 1.28 to 16.93 for NSOFC; P = 0.03, OR: 4.55 and 95% CI: 1 to 2.82 for CL; P= 0.002, OR: 7.1 and 95% CI: 1.73 to 29.16 for CLP. However, although Moasel was more frequently smoked by NSOFC fathers than those of the controls, the relationship was not statistically significant (P> 0.5). The differences between the effect of Jorak and Moasel are yet to be discovered. It could have resulted from dissimilarities in the device structure, waterpipe size, amount of tobacco consumed, and/or the device heating temperature. There could also be variation in the intensity of toxic content and consumption between the two devices. Further research on the effect and variations between the two waterpipe devices are required.

Consideration of the type of smoking device as a potential cofactor provides important information regarding the aetiology of NSOFC and further support to the argument for a smoking dose-response relationship. Although generally paternal smoking showed no increase in CL in our study, those who reported smoking were mainly waterpipe smokers (11 (64.7%) of 17 CL smoking fathers compared to 15 (16.7%) of 90 control smoking fathers) and heavy smokers compared to controls (71.4% of those reporting their smoking frequency were heavy smokers compared to 47.8% of the controls).

In 2014, the Surgeon General's Report highlighted a wide range of acute and chronic adverse health effects in infants and increased risk of adverse health outcomes resulting from second hand smoking. Our study found more frequent CL mothers (77.8%) reporting 2nd-hand smoking exposures from their husbands compared to controls (47%). Thus indicates that although the prevalence of paternal smoking is more frequent in controls compared to CL cases, they were avoiding smoking in front of their families. Moreover, there was significantly more mothers with smoking husbands in CLP and CP reported being exposed to 2nd-hand smoke compared to controls (OR: 10.54 and 95% CI: 2.29 to 48.64; and OR: 15.23 and 95% CI: 1.9 to 122.3 respectively) as most of the mothers included in our sample are housewife's (86.5%).

Maternal passive smoking has been associated with NSOFC in several other studies in addition to the current study (Jia et al., 2011; Li et al., 2011; Zhang et al., 2011). This association was confirmed in our systematic review and meta-analysis for the effect of passive smoking on oral cleft (Sabbagh et al., published/ in press 2015) (Appendix B2). Passive smoking has also been suggested to be the reason underlying why NSOFC remains a constant issue despite a decline in maternal smoking levels (Bille et al., 2005; Chevrier et al., 2008). Although we found no significant relationship between passive smoking and NSOFC

for the total sample, there was a trend towards an association, particularly for CLP (P= 0.088, OR: 1.68 and 95% CI: 0.93 to 3.06) and CP (P = 0.231, OR: 1.52 and 95% CI: 0.76 to 3.03). In addition, the analysis for two of the cities (Jeddah and Maddina) presented in "Environmental risk factors in the aetiology of non-syndromic orofacial clefts in the Western Region of Saudi Arabia" (Appendix B7) demonstrated a significant relationship between passive smoking and NSOFC (P= 0.05, OR: 2.05, 95% CI: 1.05 to 4.01).

Therefore, paternal smoking could be assumed to be a more public health concern in considering the risk of NSOFC as it is not only found to be associated with oral clefts but also is more common than maternal smoking. However, its effect depends on the intensity of smoking, device used and the existence of maternal passive smoking exposure. Further studies including larger sample sizes and smoking intensity measurements are warranted to confirm these issues. Also, investigating the effect of waterpipe on infant development is strongly recommended.

6.3.1.11 Maternal domestic exposure (Table 5.35 to 5.38)

During the pre-gestation period, a significant relationship between CLP and maternal reporting of exposure to chemicals was reported (P= 0.003, OR: 2.23 and 95% CI: 1.3 to 3.82) as well as in the 1st trimester period (P= 0.043, OR: 1.74 and 95% CI: 1.02 to 2.99) and also with exposure to solvents (P= 0.011, OR: 2.25 and 95% CI: 1.18 to 4.28). These findings are supported by similar results from several other studies (Lorente et al., 2000; Chevrier et al., 2006; Garlantézec et al., 2009; Alsahafi, 2010; Desrosiers et al., 2012). The effect of exposure was greatest in the pre-gestation period, as were our findings regarding the ingestion of maternal antibiotics and maternal illness discussed previously.

To the best of our knowledge, this is the first study to identify an association between the occurrence of NSOFC and both maternal exposure to incense and the source of maternal drinking water. This is an important preliminary observation and, if confirmed in future studies, raises the possibility of community prevention programs in the future through modification of the mineral content concentration of water and incense exposure in air in various enclosed areas. The relationship between maternal water supply and NSOFC may be related to the concentration of minerals in water, with a greater mineral content in Zamzam water compared to in either tap or bottled water. Zamzam contains the highest comparable amount of several minerals, including calcium, fluoride, zinc and magnesium (Alfadul and Khan, 2011; Zamzam Studies and Research Centre, 2011; Al Zuhair and Khounganian 2012; Shomar, 2012). Two of these minerals (zinc and calcium) were previously reported to have a significant association with decrease chance of having an infant with NSOFC (Tamura and Goldenberg, 1996; Krapels et al., 2004b; Munger et al., 2009; Hozyasz et al., 2009; Jia et al., 2011). In addition, Ruckart et al. (2013) conducted a unique study that was the 1st to suggest a relationship between contaminated drinking water and both neural tube defects (NTD) and oral cleft in their case-control study in North Carolina. They found a significant relationship between NTD and contaminated water, but the finding was not significant for oral clefts. However, because their sample size was small, further studies should be conducted to confirm their findings.

While our study has demonstrated a reduced risk of CL with maternal exposure to incense in the 1st trimester (P= 0.005, OR: 0.47 and 95% CI: 0.28 to 0.8), it is unclear what the underlying protective mechanism, if any, might be. There is however other evidence of incense's beneficial effect which may result from the putative antibacterial effects of burned incense on the surrounding air as reported by Twort and Baker (1940) and Bevilacqua et al.

(1997)). In one study carried out in Hong Kong, looking at the confounding effect of the association between air pollution and female lung cancer, although incense was found to have no effect on lung cancer risk among non-smokers, it significantly reduced the risk of cancer in smokers (P= 0.01) (Koo and Ho, 1996). No explanation was offered on how this effect might be mediated. Future study is required to confirm these preliminary findings and suggestions.

6.3.1.12 Multiple logistic regression analysis (table 5.39):

Variables that were found to be significant following logistic regression analysis and increased CL/P and/or CP risk included: maternal common cold/flu infection three months pre-gestation; maternal ingestion of antibiotics in the pre-gestation and 1st trimester periods; maternal stress; and paternal waterpipe smoking. On the other hand, folic acid supplementation, antipyretic medication ingestion in the pre-gestation period, maternal exposure to incense in the 1st trimester period and Zamzam as the maternal drinking water source significantly decreased CL/P and/or CP risk as determined by logistic regression analysis. Variables that were considered confounding factors in the aetiology of NSOFC were excluded by the logistic regression analysis (Appendix A30).

6.3.2 Genetic risk factors

The genetic aetiology of NSOFC worldwide remains unclear and enquiries are in the preliminary stages of exploration especially in the Middle East. Researchers have reported geographic variation in genetic risk factors (Blanton et al., 2010b; Lace et al., 2011; Mangold et al., 2011) and highlighted the need for research in this area among different populations, recommending that research with a study design as in the current investigation be performed.

This study is the first multicentre case-control study carried out in Saudi Arabia that has explored the genetic aetiology of NSOFC. Candidate genes were selected according to recent literature findings. Although *IRF6* was reported to be associated with NSOFC worldwide, in a study on the ethnic heterogeneity of the *IRF6*, Blanton et al. (2010) confirmed that the association of SNPs in *IRF6* to NSOFC varied between different ethnic groups. Thus, they supported the need for evaluation of *IRF6* variation across multiple populations to better determine its role in NSOFC (Blanton et al., 2010a). Therefore, this study aimed to investigate whether *IRF6* was also associated with NSOFC in Saudi Arabia in the Middle East. The second gene that was selected for study was *VAX1*, which is located in a region with high genome wide significance for OFC and was recently suggested to be a candidate for NSOFC trios (Beaty et al., 2010; Mangold et al., 2010)..

6.2.2.1 Hardy-Weinberg equilibrium (HWE) (Table 5.40)

Our sample was in HWE for *VAX1* rs4752028, which means that the allele and genotype frequencies in the population remained stable across generations in the absence of other evolutionary influences, indicating optimal performance of sampling and genotyping (i.e. without significant data loss). On the other hand, HWE was not met for the rs7078160 polymorphism in both cases and controls and this may be explained by the non-random mating that results from parental consanguinity (El-Hazmi 1996; Pritchard and Korf, 2008).

We recommend matching paternal consanguinity in future studies in an attempt to overcome any HWE variations between cases and controls.

6.2.2.2 Interferon regulatory factor 6 (*IRF6*) gene analysis (table 5.41 to 5.50):

Three Single Nucleotide Polymorphisms (SNPs) were selected in the *IRF6* gene and were analysed (rs2013162, rs2235375, and rs2235371). These SNPs were previously reported by several studies to have a significant association with NSOFC (Jugessur et al., 2008; Huang et al., 2009; Lu et al., 2013).

The first SNP selected was *IRF6* rs2013162. The Family Based Association Test (FBAT) showed significant over-transmission of the common C allele in NSOFC and CL/P cases (P= 014 and P= 0.018, respectively). In addition, PLINK analysis showed an association between the rare allele and NSOFC including CL/P (P= 0.016 and OR: 0.667 for NSOFC and P= 0.018 and OR: 0.644 for CL/P). A similar finding was reported by Scapoli et al. (2005), who detected an over-transmission of the common allele for rs2013162 (P= 0.004) and all haplotypes carrying these common alleles among 219 Italian CL/P trios. Similar finding was reported by Park et al. (2005). An allelic/OFC association was also supported by Blanton et al. (2005) who found a significant over-transmission of *IRF6* rs2013162 common C allele (P= 0.05) among CL/P cases. In addition, the rs2013162 rare allele reached a genome-wide significant relationship with CL/P in a study by Beaty et al. (2010). Stratification of the sample population using Asian ancestry as a factor yielded stronger evidence of association with *IRF6* rs62013162 (Beaty et al., 2010).

In contrast, Lu et al. (2013) carried out an FBAT and case-control analyses to investigate the effect of *IRF6* rs2013162 on the prevalence of CL/P in Northeast China. They reported a significantly increased risk of CL/P with the rare allele (P = 0.0001) and under transmission of the common allele in both case-control and FBAT analysis. Our findings do not support this finding which could be due to differences in the study setting and population. In our study, PLINK analysis showed significant transmission of the *IRF6* rs2013162 variants from

the paternal side (P= 0.05) rather than the maternal side (P= 0.152). This could be supported by Anderson et al. (2014) review on male-mediated developmental toxicity that suggested more frequency inherited mutated DNA from fathers compared to mothers. Brinkworth (2000) Suggested explanatory hypothesis for paternal genetic inherence effect through genomic instability or apoptosis suppression of the germ cell. However, our study found that maternal gene variants showed significant differences between cases and controls, with the more heterozygous allele genotype in CL/P mothers and the more homozygous rare allele genotype in controls mothers (P= 0.024). This suggests a maternal rare allele effect that was not previously described. Ludwig et al. (2009), in their Weinberg's log-linear model analysis on *IRF6* gene variants in Central European patients, found no difference in risk of CL/P between maternal- and paternal-driven alleles.

The other two *IRF6* SNPs that were analysed in this study were rs2235375 and rs2235371. Our research did not find any association between the transmission of rs2235375 and NSOFC nor did it find any differences between case and control allele frequencies in either SNP. However, although a statistically significant increased frequency of the paternal homozygous rs2235375 rare allele genotype (GG) and rare allele (G) was observed in controls compared to NSOFC cases in general, there was no significant relationship found with CL/P and CP. A recent case-control study in a Mexican population with 132 cases and 370 controls found a result similar to our finding. They reported no significant difference between CL/P cases and controls in the frequency of rs2235375 (P= 0.08) (Velázquez-Aragón et al., 2012). Other studies do not support the null-hypothesis for this allele, such as that performed by Huang et al. (2009) in Western China, who reported a strong association between the transmission of rs2235375 and rs2235371 (C/T) markers and CL/P. In addition, in their population-based case-control study on facial clefts in Norway, Jugessur et al. (2008)

reported a strong correlation (LD) between rs2235371, rs2235375, and NSOFC. They also reported a relationship between rs2235371 rare allele in infants and CL/P (P= 0.031, RR: 0.38 and 95% CI: 0.16 to 0.92). Furthermore, Scapoli et al. (2005) detected over-transmission of the common C alleles for rs2235375 (P= 0.002). Moreover, in a hospital-based case-control study carried out in Chinese Han, Pan et al. (2009) found that the rs2235371 homozygous rare allele (TT) and heterozygous genotype were associated with decreased risk of CL/P compared to the common allele (CC). They also reported a higher risk of CL/P when two polymorphisms (rs642961 rare A allele and rs2235371 common C allele) were combined. On the other hand, a recent case-control triad study by Zhou et al. (2013), using a smaller size sample (106 cases and 129 controls) in the Chinese population, found no significant association between rs2235371 and CL/P using FBAT analysis.

The frequency of the rs2235371 rare allele was very low in our study, which made it irrelevant to carry out FBAT analysis. However, this low number was supported by the report of the Ensemble Genome project, which produced a genome database that calculated the prevalence of the rare T allele in rs2235371 to be only 2% in the European population compared to 41% in the Asian population.
(http://asia.ensembl.org/Homo_sapiens/Variation/Population?db=core;r=1:209963580-209964580;v=rs2235371;vdb=variation;vf=1807176). Similarly, in their four population case-parent trios, Park et al. (2007) found that the rare allele frequency of rs2235371 in European Americans was too low to be reported. Therefore, to verify the relationship between rs2235371 and CL/P in the Saudi population, a much larger sample size would be required.

Our study did not find any relationship between *IRF6* and CP. This finding is supported by other studies that investigated the relationship between NSOFC and *IRF6* and included CP cases in their sample. They also found no significant relationship between *IRF6* and CP

(Huang et al., 2009; Jugessur et al., 2008; Scapoli et al., 2005). Other studies for *IRF6* exist as well, but these did not include CP cases in their sample.

In conclusion, the differences between our findings and previous studies could indicate that the Saudi population has different genetic aetiology of NSOFC than elsewhere in the world. A well designed GWAS study is recommended to clarify genetic aetiology of NSOFC.

6.2.2.3 Ventral anterior homeobox 1 (*VAX1*) (table 5.51 to 5.58):

Our study showed that although FBAT analysis did not show a significant over-transmission of the *VAX1* SNP alleles in NSOFC cases, there were significant differences in the frequency of genotypes and minor alleles between NSOFC cases and control infant-parental triads. Moreover, the prevalence of the parental rare allele in NSOFC cases for both rs7078160 and rs4752028 were significantly more than controls, indicating a parental effect. An association between *VAX1* and NSOFC was supported by Mangold et al. (2010) in their GWAS study of Central Europeans, reporting that rs7078160 reached a statistical significance at whole genome level. In addition, Beaty et al. (2010) found that *VAX1* rs7078160 approached genome-wide significance in Transmission disequilibrium test (TDT) and conditional logistic regression.

Our *VAX1* association finding was further supported by the results of Butali et al. (2013) in their replication of GWAS signals on 651 case-parental triads (Asian (494 infant-parental triads) and European (157 infant-parental triads) populations). FBAT analysis reported a statistically significant strong association in the transmission of the *VAX1* rs7078160 SNP among the Asian population ($P< 0.001$). However, a *VAX1* association was not significant for the European population, similar to our findings. Moreover, Butali et al. (2013) compared cases with controls in the Asian population and found an increased frequency of the common

variant (G) compared to controls, which differs from our findings. Such differences could indicate ethnic and geographic variation between the Saudi population, considered Caucasians, and the Asian population in the genetic aetiology of NSOFC

Our finding differed from those of Nasser et al. (2012), who reported no significant association between NSOFC and *VAX1* in a case-control study. However, the SNPs they sequenced did not include the two variants included in this study. In addition, Nasser et al. (2012) did report the possibility of a relationship between *VAX1* and OFC.

VAX1 rs4752028 was the only SNP examined that showed association with CP compared to controls (P = 0.049 for the fathers, P = 0.015 for the mothers and P = 0.009 for infants). Butali et al. (2013) reported no significant association between rs4752028 and CP. However, their finding was concluded from TDT (FBAT) analysis and not from a case-control design. As *VAX1* is a recently discovered gene in terms of risk for CL/P and CP, studies that clarify the relationship between NSOFC and *VAX1* are still needed.

6.2.2.4 Infant *VAX1* and parental consanguinity (table 5.59 to 5.60):

VAX1 has been reported to be associated with OFC in consanguineous marriages (Slavotinek et al., 2012) and by this current research project (P= 0.04) (Appendix A31). The relationship between *VAX1* (rs7078160 and rs4752028) and consanguinity in case compared to control infants were analysed. For rs4752028 SNP, the rare allele was found more often in CL/P cases with consanguineous parents compared to controls (P= 0.001, OR: 2.97 and 95% CI: 1.54 to 5.76).

In addition, VAX1 rs7078160 rare A allele was more frequent in CL/P cases with consanguineous parents compared to control (P= 0.081, OR: 1.93 and 95% CI: 0.92 to 4.04). That could explain the significant association of this gene in the Saudi population with the

high prevalence of consanguineous marriages. If these rare alleles are found to be associated with NSOFC it should encourage counsellors to advice against consanguineous marriages.

6.2.2.5 Haplotype based association (table 5.61):

This research found two significant haplotypes. The 1st significant blocks included *IRF6* rs2235371 and rs2013162 common alleles, which was similar to the findings of Park et al. (2007) who reported that the rs2013162 and rs2235371 common C allele haplotypes were associated with increased risk of CL/P in all their included four populations (77 European Americans, 146 Taiwanese, 34 Singaporean, and 40 Korean) (Park et al., 2007). It also included VAX1 rs4752028 rare C allele. On the other hand, the other significant haplotype block in our study included an *IRF6* rs2013162 rare allele. Further investigation for significant haplotypes is recommended in future population-based study.

6.3.3 Gene-environmental interaction

Only a few studies have considered gene-environmental interactions (GEI) in the aetiology of NSOFC. The factors that were studied were mainly folic acid and vitamin supplements, smoking and maternal passive smoking. Furthermore, only a small number of gene variants were analysed, including *IRF6* but not *VAX1* (Chevrier et al., 2008; Krapels et al., 2008; Shi et al., 2008; Wu et al., 2010). A role for *IRF6* interaction with environmental factors in the aetiology of NSOFC, however, still needs further clarification as Wu et al. (2010) did find evidence for an *IRF6* gene-environmental effect with maternal multivitamin supplementation and environmental tobacco smoke.

Studies have different scopes to consider when evaluating GEI in the aetiology of NSOFC; the effect of environmental exposures on the infant's genes and/or the interaction between environmental exposures and maternal genes influence the intra-uterine environment, resulting in favourable or unfavourable surroundings in which the foetus develops (Shi et al. 2008). In our study, we focussed on the interaction between environmental exposures and maternal genes. To confirm the existence of significant reaction between the included maternal and infant gene SNPs, log-linear model was carried out (see appendix A37). . However, this finding is different from the PLINK findings, which did not find a parent of origin effect from mother side at all. This is because the PLINK measured the transmission effect. But, the log-linear measured the direct maternal effect that could occur during pregnancy in the uterus.

One of the public health concerns in the GEI studies is the study power. False-positive and false-negative outcomes were reported in studies with small sizes (Dempfle et al., 2008). To improve the reliability of our results, two study designs and three statistical analyses were carried out for GEI. A case-only study design evaluated maternal SNP genotype variant (homozygous common allele genotype, heterozygous allele genotype and homozygous rare allele genotype) and allele types (common vs. rare) and their interaction with environmental factors. A case-control study design was used to evaluate the association between maternal SNP genotypes and environmental factors. The gene-environmental interactions that were confirmed by both study designs and/or multi-nominal logistic regression analysis (table 5.62), are given more consideration.

Moreover, because only a minimal number of rare allele genotypes for *IRF6* rs2235371 were observed in our sample, the attempt to investigate any interaction between this SNP and

environmental factors produced values that were similar to those produced by analysing environmental factors alone. Therefore, we did not carry out further analysis for this SNP.

6.3.3.1. Gene-environmental interaction between maternal *IRF6* rs2013162 and environmental risk factors (Appendix A38 to A40)

The environmental risk factor that showed a significant interaction with maternal *IRF6* rs2013162 in both study designs and statistical approaches used in this research was maternal folic acid supplementation during the pregestation period. The analysis showed that mothers with an *IRF6* rs2013162 homozygous rare AA genotype and rare A allele that ingested folic acid supplementation in the pregestation period were statically significant more likely to have a child with NSOFC compared to those with common allele genotype (OR: 8.22 and 95% CI: 1.03 to 65.72 for AA compared to CC; and OR: 2.57 and 95% CI: 1.23 to 5.37 for A compared to C). In addition, in our case-control design, 18% of NSOFC mothers with the homozygous rare AA allele genotype consumed folic acid supplementation during the pregestation period when no mothers reported using it in the control group (P= 0.023). This indicates a synergistic effect that increases the risk of NSOFC, when using folic acid in the pregestation period. The significant association follow a recent population base case-control study in Northern Netherlands that reported a "duration of exposure-response effect" that increased the risk of cleft lip (Rozendaal et al., 2013).

Moreover, the maternal homozygous rare AA genotype was reported in this study to be significantly less frequent in cases compared to controls. In addition, folic acid supplementation was thought to decrease the risk of cleft. Therefore, mothers with the common C allele that are exposed to folic acid during the pregestation period are less likely to have a child with NSOFC. Finally, although wide confidence interval reported in the case-

only genotype analysis may indicate insufficient sample size, confirmation of the relationship was repeatedly observed in the different statistical analysis methods. In contrast, in the case-control study design, maternal folic acid in the 1st trimester period were significantly more ingested by the controls with homozygous rare AA allele genotype mothers compared to cases (P= 0.003), indicating a suggested protective joint effect.

Other environmental risk factors were either significant in the case-only study design or in the case-control study designs. In the case-only study design, antipyretic medication and fever showed GEI in both analysis (genotypes and allele analysis). Mothers with rare allele (A) and homozygous rare AA allele genotype using antipyretics in the 1st trimester period were significantly more likely to have an infant with NSOFC (OR: 9 and 95% CI: 1.62 to 49.91 when the AA genotype is compared to CC; and OR: 2.34 and 95% CI: 1.12 to 4.9 when the A allele was compared to the C allele). This could also indicate that mothers with homozygous common CC allele and using antipyretics during the 1st trimester period are significantly less likely to have an infant with NSOFC. In addition, maternal fever in the pregestation period was significantly less common in mothers with the homozygous AA rare allele genotype (OR: 0.26, and 95% CI: 0.08 to 0.84) and rare A allele (OR: 0.28 and 95% CI: 0.09 to 0.81) among NSOFC mothers, indicating that mothers with common homozygous CC genotype variant that are exposed to fever are significantly more likely to have an infant with oral cleft. These relationships suggest a synergic effect of environmental risk factors that influence the effect of the maternal common allele. It also highlights the importance of future studies aimed at verifying the influence of maternal disease symptoms on the function of maternal genes and their effect on the embryonic development.

Moreover, in the case-control study, mothers who were homozygous for the common allele (CC), and consumed antibiotics during the pregestation period or were exposed to illness and

common cold/ flu were significantly more likely to have an infant with NSOFC. In addition, paternal waterpipe smoking when associated with maternal common allele (CC) they are more likely prone to have an infant with NSOFC. This indicates a synergic effect between environmental risk factors and gene variant that when isolated were also shown in this thesis to be associated with an increased risk of oral clefts.

Other environmental factors that showed a significant interaction with the homozygous rare allele (AA) but only in one of the analysis were multivitamins and iron supplementations; x-ray exposure; maternal smoking, maternal passive smoking and; maternal stress; abdominal pain, maternal exposure to chemicals and maternal drinking water (Bottle and Zamzam drinking water source vs. tap water).

Nevertheless, these associations highlight the possibility of developing a prevention program. Future studies including larger sample sizes are needed to clarify the relationship, interactions and different supplementation doses.

6.3.3.2. Gene-environmental interaction between maternal *IRF6* rs2235375 and environmental risk factors (Appendix A41 to A43)

The environmental risk factor showing a significant interaction with maternal *IRF6* rs2235375 in both study designs and all GEI statistical approaches used in this study was maternal stress. Our analysis showed that mothers with the *IRF6* rs2235375 homozygous rare GG allele genotype and rare G allele that are exposed to stress during the pregestation and 1st trimester periods were statistically significant less likely to have a child with NSOFC (P= 0.016, OR: 0.3 and 95% CI: 0.1 to 0.8 in genotype analysis; and P= 0.01, OR: 052, 95% CI: 0.32 to 0.84 in allele analysis). It also indicates that mothers with the homozygous common C allele that were exposed to stress are more likely have an infant with NSOFC. In

addition, the case-control study design showed a significant interaction between stress and NSOFC mothers with the homozygous common allele (CC), who were significantly more likely to be exposed to stress compared to controls (P= 0.014).

An interaction between folic acid supplementation in the pregestation period and maternal *IRF6* rs2235375 was found to be associated with NSOFC. The case-only study design showed that mothers with the homozygous rare allele (GG) and ingested folic acid supplementation in the pregestation period were significantly less likely to have an infant with NSOFC compared to those with the homozygous common allele (CC) (P= 0.045, OR:0.23 and 95% CI: 0.05 to 0.97). Case-control analysis also revealed statistically more NSOFC mothers with the homozygous common allele (CC) that ingested folic acid supplementation in the pregestation period compared to controls (P= 0.026). However, this finding was not supported by Velázquez-Aragón et al (2012) study that was carried out in Mexico did not find an interaction between *IRF6* rs2235375 and folic acid in the aetiology of oral clefts. However, the differences in the results may be related to differences in study setting

Although multivitamins supplementations interaction with *IRF6* rs2235375 was significant in the case-control analysis (P= 0.026) it was not supported by Wu et al (2010) population based study that was carried out in China. In addition, the number of subjects exposed to multivitamins in our study was small and the significant relationship was only found in one of the analysis and in the pregestation period only. Therefore, the relationship is only a preliminary report.

Other environmental factors showing a positive interaction with the homozygous common allele but were only significant in one of the analysis included maternal fever, illness,

common cold/flu, x-ray exposure, paternal waterpipe smoking and consanguinity. Future studies will clarify the relationship we presented in this study.

6.3.3.3. Gene-environmental interaction between maternal *VAX1* rs4752028 and environmental risk factors (Appendix A45 to A47)

The environmental risk factor showing a significant interaction with maternal *VAX1* rs4752028 in both study design approaches used was maternal fever. The analysis showed that mothers with the *VAX1* rs4752028 homozygous common T allele, which were considered to decrease the likelihood of NSOFC, that were exposed to fever were statistically significant more likely to have a child with NSOFC (for allele analysis: P< 0.001, OR: 0.11 and 95% CI: 0.05 to 0.26 in the 1st trimester period; and for case control: P= 0.03, OR: 2.79 and 95 % CI: 1.07 to 7.25 in the pregestation period, and P= 0.053 in the 1st trimester).

This interaction between maternal genotype which was considered to decrease the chance of having an infant with NSOFC ; with an environmental risk factor that was previously suggested to increase the risk of NSOFC, could indicate a strong environmental risk factor that can overcome the effects of the genetic factors. Another explanation may be that although these genotypes were expected to protect the individuals from NSOFC, when exposed to negative environmental factors, they contributed to the opposite effect. Alternatively, putative conflicting effects indicate that other, potentially related genes are involved in the interactive pathway, or that a relevant gene-gene interaction remains unknown.

Mothers with the homozygous rare allele genotype (CC) that had high blood pressure during the 1st trimester were significantly more likely to have an infant with oral cleft compared to

those with the homozygous common allele (TT). Other environmental factors that were more likely to increase the occurrence of NSOFC offspring in mothers with homozygous common allele (TT) were antibiotics ingestion, illness, common cold/flu infection, multivitamins and stress. Mothers with the homozygous common allele (TT) that ingested folic acid were less likely to have NSOFC infant. Finally, incense showed a reduced chance of having an infant with NSOFC when interacting with the homozygous rare allele, which is considered to increase the risk of NSOFC.

6.3.3.4. Gene-environmental interaction between maternal *VAX1* rs7078160 and environmental risk factors (Appendix A48 to A50)

None of the environmental risk factor showed significant interactions with maternal *VAX1* rs7078160 in both study designs approaches and analysis together. However, in the case-only study design analysis, paternal waterpipe showed a significant interaction with the *VAX1* rs7078160 homozygous rare allele (AA) and rare allele (A). The analysis showed that mothers with the *VAX1* rs7078160 homozygous rare allele (AA) and rare allele (A) were considered to increase the likelihood of NSOFC, and those exposure to paternal waterpipe smoking were statistically significant more likely to have a child with NSOFC (P= 0.016, OR: 5.54 and 95% CI: 1.38 to 22.23 for the genotype analysis; and P= 0.01, OR: 2.67 and CI: 1.28 to 5.56 for allele analysis). The interaction between smoking and genetic variants has been studied previously. In 2008, Shi et al. reviewed studies that investigated the interaction between genetic variants and maternal smoking in the aetiology of oral cleft and reported that GEI studies were still in early stages. In addition, paternal smoking including types of smoking and the *VAX1* gene were not previously investigated (Shi et al., 2008).

Other environmental factors in case-control study that showed a significant positive interaction with the *VAX1* rs7078160 homozygous common allele (GG), which was considered to be associated with a decreased risk of NSOFC are; maternal antibiotic ingestion, illness, common cold/flu and family history. Maternal exposure to chemicals in the pregestation period was significantly less associated with NSOFC mothers with rare A allele compared to those with the common G allele. This indicates that mothers with the common allele G and exposed to chemicals were more likely to have a child with oral cleft. Other environmental factors showing a significant positive interaction with *VAX1* rs7078160 homozygous rare AA genotype and rare A allele in case-only study included high blood pressure in the 1st trimester and paternal consanguinity. It is important to carefully address the synergic effect between environmental and genetic factors for future public health intervention.

To draw conclusions regarding a definitive gene-environmental interaction, a larger sample size is necessary in a GWAS study using a log-linear modelling approach. However, obtaining the number of cases for an adequate sample size for genetic analysis and environmental risk factor exposures is difficult, particularly because NSOFC is a rare disease and sub-phenotyping is necessary. In addition, there are multiple risk factors that contribute to these diseases (Zhu et al., 2009; Hutter, 2013). The value in preliminary studies that may be underpowered for definitive findings lies in them being valuable instruments for preliminary description of GEI and for generating hypotheses that can be tested with adequate power (Dempfle et al., 2008; Hutter, 2013). Accordingly, we expect that our findings will play an important role in directing and assessing future research to identify

possible gene-environmental risk factors that may have a role in prevention of NSOFC through public health strategies.

This study shows many risk factors; genetic, environment and gene-environmental interaction that are suggested to play a role in the aetiology of oral clefts in Saudi Arabia. However, the aetiological risk factors that we analysed in this study could be of real importance to the Saudi population if the population attributable risk (PAR) was high. However, In order to assess PAR we need to analyse and compare our data with the prevalence of exposures to these risk factors in the Saudi population. The prevalence of exposures to some of these environment risk factors among the Saudi population were somewhat possible when found in the literature. For example; the prevalence of smoking (Bassiony, 2009; Fida & Abdelmoneim, 2013), diseases such as diabetes (Khwaja et al., 1989; Ardawi et al., 2000; Al-Hakeem, 2006), hypertention (Al-Ghamdi et al., 1999) and heart diseases (Ministry of Health, 2009), and paternal consanguinity (Elhazmi et al., 1996). However, other factors such as the prevalence of pregnant mothers using supplementations, antibiotics, or exposed to stress and diseases among the Saudi population are not found in the literature. In addition the incidence of exposure to environmental factors in the population was not always specific to pregnant woman which makes it difficult to estimate PAR. In addition, genetic mapping specifically designed for Saudi population does not yet exist.

Therefore, future research that aims to affirm NSOFC risk factors relationship; calculate the risk of environmental exposures in the population; genotype the population DNA; and measure the population attributable risk, is important for public health prioritization, approaches and strategies designed to prevent and control NSOFC in the Saudi population

6.4 Strengths and Limitations

6.4.1 Strengths

- Geographic and ethnic variations have been shown to play a part in influencing the aetiology and prevalence of NSOFC (Mossey at al.,2009). There has been little investigation into these factors and their genetic and environmental origins in the Middle East, and therefore the study is a useful contribution to addressing significant data gaps.

- Previously studied risk factors such as consanguinity and dietary factors have also been explored in this study, and the unique combination of factors makes Saudi Arabia, the largest country in the Middle East a useful part of the world for studying risk factors in NSOFC.
- Some factors, not previously investigated, including maternal drinking water supply and different types of tobacco smoking have emerged as a result of this careful investigation as possible risk factors, and these should be explored further in future studies.
- The sample included three main cities in Saudi Arabia that covers almost 60% of the population according to the Ministry of Health.
- To try to obtain as comprehensive ascertainment as possible, we pooled most cleft cases living in Jeddah and Maddina: Jeddah city was divided geographically into five districts. We included all governmental referral hospitals for NSOFC in Jeddah city, distributed across the five districts, in this study. In Maddina, the only referral center, which was also the main maternal children hospital in Maddina city, was included.

Therefore we have made efforts to include as many of the NSOFC cases in the two cities as possible.

- The study is novel as it is the first study that describes the birth prevalence of NSOFC and its phenotypes in three cities of Saudi Arabia.
- The study was carried out in a population with high prevalence of paternal consanguinity which provided good baseline numbers to examine the effect of consanguinity although the type of consanguineous unions in the Middle East is different from those in other parts of the world, for example India.
- This case-control study is the first, to the best of our knowledge, to investigate the environmental risk factors for NSOFC in the Western and Central Region of Saudi Arabia.
- It is the first study to look into the association between the different type of tobacco smoking devices and both CL/P and CP.
- Water-pipe smoking is considered an emerging global epidemic concern that is largely understudied (Fakhreddine et al. 2014). This paper provides further evidence of the need to study the association between different smoking exposure devices and mechanisms including water-pipe and passive smoking in relation to congenital anomalies.
- It is the first study to look into the association between the maternal drinking water source and both CL/P and CP.
- This study investigate VAX1 which was recently examined and IRF6 gene, and it is relationship to CL/P and CP for the 1st time in Saudi Arabia

- One of the strengths of this study is investigating VAX1 gene in a population with a high prevalence of paternal consanguinity.
- The study presents preliminary information on gene-environmental interaction in the aetiology of oral clefts.

6.4.2 Limitations

There were some limitations to this study that should be considered:

- Although we expect to have included almost all cases in Jeddah and Maddina, we were not able to include King Faisal Hospital and research centre in Riyadh which is considered an important cleft centre. However, this does not affect part I of our study (prevalence assessment), because King Faisal Hospital and research centre is not a maternity hospital.
- Stillbirths were not included in this study, which may have caused bias in assessing the prevalence of OFC (Al-Omari and Al-Omari, 2004; Welch and Hunter, 1980). However, stillbirth prevalence is expected to be low, accounting for only 15.7 in every 1000 births recorded by the Ministry of Health, Saudi Arabia (Ministry of Health, 2010).
- Recall bias was considered to have minimal effect on our findings according to previous studies ((Khoury et al., 1994; Swan et al., 1992). Also, it could be overcome through increasing of the proportion of exposed controls (Poletta et al., 2012)

- Bias could have occurred from questions related to smoking. This could have resulted from social stigma associated with smoking. However, this attitude is expected from parents of both cases and controls.
- The attitude toward questions related to family problems and the different in maternal coping with stress are expected. However, it is anticipated in both groups (cases and controls)
- The frequencies of maternal exposure to some of the environmental aetiological risk factors were inadequate to produce a definitive conclusion for factors such as maternal smoking, type of smoking device, and maternal water drinking source. Therefore, a larger sample size is needed to confirm our findings. However, these factors were still discussed because of their importance in future studies and their significance in the development of community preventive programs.
- There is a possibility that some of the associations found in this study between NSOFC and exposures have arisen from chance (the probability of statistically significant relationship arising by chance alone is one in twenty for each tested exposure) (Scialli, 2014).
- There was potential for confounding factor effects, which we tried to overcome by carrying out logistic regression analysis.
- Cases and controls were recruited from medical centres located solely in urban areas.
- The sample did not meet Hardy-Weinberg equilibrium in some situations.
- The sample size was not sufficient to draw a final conclusion for the gene-environmental interaction (GEI) or to investigate the role of GEI in NSOFC sub-phenotypes (CL/P and CP).

Chapter 7: Conclusion and future plans

7.1 Conclusions

This study was carried out in three main cities in Saudi Arabia from January 2010 to January 2012 to measure the prevalence and aetiology of non-syndromic orofacial cleft. We report the following conclusions:

7.1.1 Prevalence of NSOFC and the influence of parental consanguinity on CL/P and CP in Saudi Arabia

Saudi Arabia and some Middle Eastern countries do not have a baseline data on orofacial cleft prevalence which would be essential pre-request information for any intervention. This multicentre study assessed the prevalence of NSOFC and its phenotype in three main cities in Saudi Arabia:

- The prevalence of NSOFC in Saudi Arabia (1.17/1000 live births) was found to be marginally lower than the global average figures (1.25/1000 live births).
- The prevalence of NSOFC was found to be highest in Maddina (1.88/1000 births), followed by Riyadh (1.07/1000 live births), with the lowest prevalence in Jeddah (0.81/1000 live births).
- The overall mean birth prevalence of CL/P was found to be 0.89/1000 births across all three cities.
- The prevalence of CL (0.47/1000 live births) was found to be higher than that of CLP (0.42/1000 live births) and CP (0.28/1000 live births).

- The prevalence of consanguinity in NSOFC infants was found to be 81 (65.9%) out of 123 cases. This value was higher in CP than in CL/P, and higher in severe CL/P (complete clefting of the lip or bilateral cleft), but the difference was not significant.

7.1.2 Environmental risk factors associated with CL/P and CP in Saudi Arabia

Maternal exposure to common cold/flu, folic acid supplementation, stress, antibiotic ingestion, chemicals, incense, source of drinking water and paternal waterpipe are associated with increased / or decreased risk of CL/P and/or CP in Saudi Arabia

- There was no significant relationship between demographic variables and both CL/P and CP in Saudi Arabia.

- There was no significant relationship between socioeconomic status and both CL/P and CP in Saudi Arabia, except for residency description, and there are significantly more CP cases live in rural areas than there are controls.

- Family history of oral cleft was found to be significantly associated with increased risk of CL/P and CP in Saudi Arabia

- Maternal common cold/flu infection during the pre-gestation period were found to be associated with an increased risk of CL/P and CP in Saudi Arabia

- Maternal antibiotic ingestion in the pre-gestation and 1st trimester period were found to be associated with an increased risk of CL in Saudi Arabia.

- Folic acid supplementation in the 1st trimester period was found to be associated with a decrease risk of CLP in Saudi Arabia.

- Maternal stress was found to be significantly associated with an increased risk of CL/P and CP in Saudi Arabia.

- Paternal waterpipe smoking and intense paternal smoking were found to be significantly associated with an increased risk of CL/P and CP, in Saudi Arabia.

- Maternal exposure to chemicals during the pregestation period was found to be significantly associated with an increased risk of CLP in Saudi Arabia.

- Maternal exposure to incense appears to influence CL risk.

- Source of drinking water appears to influence oral cleft risk. Maternal drinking water that contains higher amount of minerals such as zinc, calcium, or magnesium, and is alkaline was found to decrease the chance of having an infant with NSOFC.

- Other maternal exposures such as anti-pyretic medication, anti-emetic medication, fever, passive smoking, duration of inter-pregnancy interval, and x-ray were significantly associated with CL/P and/or CP, but were not found to be significant in the logistic regression analysis.

The above findings raise the possibility of introducing community preventive programs through changing a range of behavioural and lifestyle factors or altering the mineral content of drinking water.

7.1.3 Genetic risk factors associated with CL/P and CP in Saudi Arabia

To the best of our knowledge, this is the first multicentre study that investigates the genetic aetiology of NSOFC in Saudi Arabia. *IRF6* rs2013162 showed significant over transmission of the common allele (C) with CL/P cases. Also, *VAX1* rs4752028 and rs7078160 rare allele

are found more frequent in CL/P and CP infant-parental triad cases compared to controls except for paternal rs7078160 rare homozygous allele.

- The transmission disequilibrium test (TDT) and PLINK analysis showed that the *IRF6* rs2013162 rare A allele is not a marker for CL/P risk as the common C allele (C) was the one showing significant transmission in CL/P cases.
- Parent of origin was found to be significant for the *IRF6* rs2013162 SNP, which showed significant over-transmission of the *IRF6* variant from the paternal side in NSOFC cases. But, it was not significant when subdividing NSOFC to CL/P and CP.
- There was a significant difference found between CL/P cases and controls for the maternal *IRF6* rs2013162 variant, with a significantly more homozygous rare allele genotype (AA) in controls and more heterozygous allele genotype (CA) in CL/P cases.
- There are no significant differences found between CL/P and CP cases compared to controls for the *IRF6* rs2235375 and 2235371 variants.
- The *IRF6* rs2235371 homozygous rare allele genotype was found to be rarely present in the Saudi population (less than 3%).
- The *VAX1* rs4752028 and rs7078160 rare allele and homozygous rare allele were found more frequently in CL/P infant parental triad cases compared to controls, except for the paternal rs7078160 homozygous rare allele genotype.

- The *VAX1* rs4752028 rare allele and homozygous rare allele were found more frequently in CP infant parental triad cases compared to in controls, but was not significant for the maternal homozygous rare allele genotype.

- Two haplotype blocks, consisting of the five SNPs included in this study, showed a significant association with NSOFC. The first block included the common alleles of four SNPs and the rare allele of *VAX1* rs4752028, while the second block included four common alleles and IRF6 rs2013162 rare allele.

- The *VAX1* rs4752028 rare allele was found more frequent in CL/P and CP cases with consanguineous parents compared to the frequency of this SNP in controls but was statistically significant only for CL/P.

7.1.4 Genetic-environmental interaction associated with oral clefts in Saudi Arabia

The information gained from this study on GEI is valuable for public health strategies as it gives a preliminary description of GEI for two genes (*VAX1* and *IRF6*) in Saudi Arabia.

- Maternal exposure to antipyretic, folic acid, fever, antibiotics, illnesses, common cold/flu, paternal waterpipe smoking, stress, x-ray and/or chemicals could significantly interact with the maternal *IRF6* (rs2013162 and rs2235375) gene variants, affecting the risk of having a child with oral cleft.

- Maternal usage of folic acid, multivitamin, antibiotics, exposure to fever, illness, stress, high blood pressure and incense could significantly interact with the maternal *VAX1* (rs4752028) gene variant, affecting the risk of having a child with oral cleft.

- Waterpipe smoking, and chemicals are significantly associated with *VAX1* (rs7078160) variants in relationship to orofacial cleft.

- This study directs future GEI research and guide future hypotheses to be tested for confirmation in larger studies in the future.

7.2 Future plans and recommendations

This study recommends future plans and direct coming research on the aetiology of NSOFC as follow:

- Large-scale national researches program including the private sector which covers 20% of the Saudi health services (Almalki, 2011), should be considered in order to adequately and nationally assess NSOFC sub-phenotype prevalence.
- Further investigation is required to examine the influence of consanguinity on the prevalence of CL/P and CP in Saudi Arabia and the Middle East.
- The effect of less frequently exposed environmental factors with genotype should be investigated using a larger sample size and cohort study.
- The distribution of sample recruitment across both urban and rural areas, particularly for controls, should be determined in order to more accurately measure the SES and regional variation effects on the prevalence of NSOFC.
- The timing, duration, amount and relationship between folic acid and oral cleft phenotypes need to be addressed in more details
- Measures and social strategies should aim to decrease maternal stressors.
- Prospective studies that investigate the effect of different types of smoking devices, maternal smoking, and maternal tobacco second-hand exposure to different smoking devices are important for public health policies aiming for prevention.

- Prospective studies aiming to clarify the relationship between drinking water (source and content) with the risk of having an infant with CL/P and CP should be considered in the future in order to confirm the possibility of using drinking water as a method for NSOFC public health prevention
- Consanguinity could be matched between cases and controls in future genetic risk studies. A GWAS study is required to confirm and investigate genes responsible for the aetiology of CL/P and CP in Saudi Arabia and the Middle East.

- Future studies measuring the population attributable risk for the different environmental, genetic risk factors and epigenetics are required for public health planning, prioritization and strategies for the control of CL/P and CP.
- Further studies are required to determine the potential role of the interaction between environmental and genetic risk factors in primary prevention of CL/P and CP
- Further studies are required to determine the potential role of the interaction between different environmental risk factors in primary prevention of CL/P and CP. A larger sample size study involving a log-linear modelling approach is needed to draw conclusions supportive of a definitive gene-environment interaction for CL/P and CP.

- Finally, further systematic reviews and meta-analysis are required to draw a final conclusion on the risk factors attributing to oral clefts. We suggest to review the following topics:

    - SES including parental education, occupation, Description of the family neighbourhood and their relationship to CL/P and CP.

    - Maternal ingestion of Folic acid; timing and doses and their relationship to CL/P and CP.

- Maternal ingestion of multivitamins and their relationship to CL/P and CP.
- Maternal ingestion of antipyretics and exposure to fever and their relationship to CL/P and CP.
- Smoking device type, intensity and paternal smoking and their relationship to CL/P and CP.
- Parental alcohol consumption and their relationship to CL/P and CP.
- Stress and different type of stressors and their relationship to CL/P and CP.

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Chapter 9 APPENDIXES: Provided in the attached CD

Chapter 9 APPENDIXES:

Contents

Appendix A

A1 : List of Syndromes associated with OFC:

OFC phenotype	Syndrome	Gene involved
CL/P	Autosomal Dominant developmental malformation	ACTB
	Deafness and dystonia	

	Familial gastric cancer and CLP	CDH1
	Cranio-fronto-nasal	EFNB1
	Roberts	ESCO2
	Holoprosencephaly	GLI2
	Oro-facial-digital	GLI3
	Hydrolethalus	HYLS1
	Van-der-woude/ Popliteal pterygium	IRF6
	X-Linked mental retardation and CLP	PHF8
	Ectodermal dysplasia and CLP	PVRL1
	Gorlin	PTCH1
	Holoprosencephaly	SHH, SIX3, TGIF1
	Bronchio-oculo-facial	TFAP2A
	Ectrodactyly-ectodermal dysplasia-clefting	TP63
	Ankyloblepharon-ectodermal dysplasia-clefting	TP63
	Tetra-anemlia with CLP	WNT3
CP	oculofaciocardidental	BCOR
	CHARGE	CHD7
	Lethap and Escobar multiple pterygium	CHRNG
	Stickler type 1, 2 and 3	COL2A1, COL11A1, COL11A2
	Desmosterolosis	DHCR24
	Smith-Lemli-Optiz	DHCR7
	Miler	DHODH
	Craniofrontonasal	EFNB1
	Kallmann	FGFR1
	Crouzon	FGFR2

	Apert	FGFR2
	Otopalatodigital type 1 and 2	FLNA
	Larsen syndrome	FLNB
	Hereditary lymphedema	FOXC2
	Bamforth-Lazarus	FOXE1
	Andersen	KCNJ2
	Kabuki	MLL2
	Cornelia de Lange	NIPBL
	X-Linked mental retardation	PQBP1
	Isolated cleft palate	SATB2
	Diastrophic dysplasia	SLC26A2
	Ccompomelic dysplasia	SOX9
	Pierre Robin	SOX9
	DiGeorge	TBX1
	X-linked cleft palate and ankyloglossia	TBX22
	Treacher Collins	TCOF1
	Loeys-Dietz	TGFBR1 and TGFBR2
	Saethre-Chotzen	TWIST1

A2: The International Classification of Diseases (ICD) World Health Organization's (WHO) classification codes for NSOFC

ICD: Q35	**Description: CP**	**ICD: Q36**	**Description: CL**	**ICD: Q37**	**Description: CLP**
Q35.1	Cleft hard palate	**Q:36.0**	Cleft lip,	**Q37.0**	Cleft hard palate with

			bilateral		bilateral cleft lip
Q35.3	cleft soft palate	**Q:36.1**	Cleft lip, median	**Q37.1**	Cleft hard palate with unilateral cleft lip
Q35.5	Cleft hard palate with cleft soft palate	**Q:36.9**	Cleft lip, unilateral	**Q37.2**	Cleft soft palate with bilateral cleft lip
Q35.7	Cleft uvula			**Q37.3**	Cleft soft palate with unilateral cleft lip
Q35.9	Cleft palate, unspecified			**Q37.4**	Cleft hard and soft palate with bilateral cleft lip
				Q37.5	Cleft hard and soft palate with unilateral cleft lip
				Q37.8	Unspecific cleft palate with bilateral cleft lip
				Q37.9	Unspecific cleft palate with unilateral cleft lip

A3: Proposal for medical center's IRB and PI explaining the aim and procedure of the research and requesting an approval.

Investigation in the aetiology of Orofacial Clefts in Saudi Arabia

Supervising the project:

Prof. Najla Alamoudi Chairmen of Preventive Dental Sciences Department, KAU

Coordinator of the Saudi Cleft project and researcher:

Dr. Heba Sabbagh, PhD student, Pediatric Dentistry, KAU

Table of content:

Investigation in the aetiology of Orofacial Clefts in Saudi Arabia

Summary:

- Orofacial cleft is a multifactorial defect that varies in incidence geographically and within different ethnic groups. The **aim** of this study is to find out the genetic and environmental risk factors related to cleft lip and/or palate (CL/P) in infants attending governmental hospitals in the Western and Central region of Saudi Arabia prospectively using a matched case-control triads.

- **Material and methods:** Infants with non-syndromic CLP from birth to 18 months and their parents in three main cities of Saudi Arabia (Jeddah, Maddina and Riyadh) will be included. A matched control triad with the same infant's age, gender, and hospital will be chosen. Saliva samples will be collected from infants and their parents in both groups using *Oragene: DNA* (Oragene 500 for adult and Oragene 575 for infants) for selective gene analysis. A questionnaire is used to collect information about the infant's mothers and fathers three months before and during pregnancy, and family history to detect any environmental risk factors for CLP in Saudi Arabia.

- **Statistical analysis:** The descriptive epidemiology of CL/P will be presented, with statistics displayed in frequency and percentage for categorical variables, or means and standard of deviation for continuous variables. SNPs at candidate genes such as FGFR2, IRF6 will be analysed for their association with CL/P in the Saudi population. Gene-gene and gene-environment interaction will also be examined in this context.

Introduction:

Orofacial clefts consist of cleft lip with or without cleft palate (CL/P)[1] and isolated cleft palate (CP). They may present as part of a syndrome or other associated abnormalities[2]. Cleft lip and palate can lead to a series of functional as well as aesthetic problems including feeding difficulties especially at birth, swallowing and nasal regurgitation, hearing difficulties, and speech difficulties. Although these cleft defects can be surgically repaired in early childhood, residual deformity due to scarring and abnormal facial development, results in long-lasting functional and psychosocial problems [3,4]. Affected children have higher morbidity and mortality throughout life than do unaffected children.[5,6] which mean that clefts have long-lasting, adverse effects on the health and social integration of affected individuals [7,8].

Cleft lip and palate are known to be the most common craniofacial defects throughout the world. [5] It is estimated that the overall global prevalence of OFC is one individual in every 700 births. [9] However, the prevalence varies in different parts of the world and different ethnic groups. They occur 4 to 6 times more often among Asian populations but are less common in Africans. [9] Despite efforts to record the frequency of birth defects over the years, accurate data on the epidemiology does not exist in many countries [11].

In Saudi Arabia, where almost 300,000 children are born per year [12], no data on the precise prevalence of craniofacial cleft and lip anomalies are found in the dental literature. Research carried on craniofacial defects has demonstrated ranging incidence from 0.3 to 2.19 cleft infants in every 1000 live births. However, these

were hospital based studies that cannot be generalized to the Saudi population [13-17]. One pioneer project that initiated registration of cleft lip and palate anomalies was carried out in King Faisal Specialised Hospital and Research Centre. 1555 patients with cleft lip and palate and craniofacial anomaly were registered over a period between 1999 to 2008 was 774 cleft lip and/ or palate.[18,19] However, more investigations is needed on the prevalence and aetiology of OFC in order to build the foundation for controlling or even preventing OFC in the future.

The aetiology of cleft lip and palate is complex. There are many factors that contribute to cleft lip and palate; genetics, environmental, and gene-environmental interaction risk factors.[9] Genes that are involved in the aetiology of cleft lip and palate are those that are responsible for their embryonic development. However, understanding their contribution in the aetiology of cleft lip and palate is complex. The number of genes involved the differences between cleft lip and palate and isolated cleft palate [21], the heterogeneity of each group, the type of inheritance and interaction with the environmental factors makes it difficult to identify the etiology.[22] Therefore several approaches should be advocated to identify the genes; the use of a combination of family collection, careful phenotyping, high-throughput genotyping, robust analytical strategies, final structural mapping and mutation characterization. [23,24]

Genome Wide Association (GWA) is an approach that involves rapid scanning markers across the complete sets of DNA, or genomes, of many people and is used to find genetic variations associated with a particular disease. At least three genes (MSX1, IRF6, and FGFR1) appear to play a significant role in orofacial clefts.[24]

MSX1 is located on chromosome 4p. It is one of the genes responsible for initiation and growth of the facial processes and specification of its identity.[25] The mutation of this gene is responsible for 2% of nonsyndromic cleft lip and palate.[26,27] The gene that encodes the transcription factor interferon regulatory factor 6 (IRF6) is located on chromosome 1q. It is related to the formation of connective tissue in the palate. Studies have found that mutations in this gene results in the autosomal dominant disorder Van der Woude syndrome (VWS).[28] VWS resembles an isolated cleft, but is accompanied in most cases by lip pits, caused by mutations in a single gene, whereas the more common isolated cleft is a complex trait caused by multiple gene mutations and/or environmental insults. Very recently it was demonstrated that a common haplotype associated with IRF6 contains a mutation that provides a risk of approximately 12% to all common forms of cleft lip and palate. [24]

The other gene, FGFR1 was identified on chromosome 8p [29] in cases of Kallmann syndrome, an autosomal dominant disorder typically characterized by infertility and anosmia. Approximately 5% of Kallmann syndrome cases have clefts of the lip and/or palate and, as with VWS, some individuals may present with clefts as the only component of the phenotype.[30]

Epidemiological and experimental evidence suggest that environmental risk factors such as maternal exposure to tobacco smoke, alcohol, poor nutrition, viral infection, medications, and teratogens in the workplace and at home in early pregnancy are important factors in aetiology. [24,31]

Consanguinity has been reported to be higher in parents with orofacial cleft children than non-cleft children.[32] It was suggested that this could play a role in the aetiology

of clefts. Saudi Arabia has a high prevalence of consanguinity (57.7%)[33] and it would present an opportunity to carry out such an investigation. In King Faisal Hospital and Research Centre [19] they found that 53.3% of the cases were of first cousins which is rather a high rate compared to the prevalence of 1st cousin consanguineous marriages reported in the general Saudi population.[33] On the other hand, Alsahafi Y (2010) concluded in his study on the association between orofacial clefts and consanguinity in Saudi Arabia, that it had a protecting effect against having a cleft child.[34] Therefore, further investigation is needed to identify the effect of consanguinity on oral clefts.

The presence of risk factors, if identified, could be manipulated to control or reduce the incidence of the anomalies. Therefore, studying and understanding the aetiology of cleft lip and palate is significant to prevent and limit the occurrence of these defects in the future.

This study **Aim** is to investigate the prevalence and aetiology of cleft lip and/ or palate in the western and central region in Saudi Arabia.

Material and Method:

The proposed prospective study is designed to investigate the risk factors related to cleft lip or/and palate using parents-child trios in the central and western regions in Saudi Arabia.

Subjects:

The study will take place in three cities in Saudi Arabia which contain most of the Saudi population (Riyadh, Jeddah and Maddina).

The government hospitals in these areas, as described by the ministry of health book (2006), [52] includes the hospitals of the Ministry of Health, University Hospitals, National Guard Hospitals, and King Faisal Specialised Hospitals and research Centres. In each city the sample will be stratified according to geographic location to five districts (central, north, south, west and east). Random sample of hospitals will be selected according to proportional allocation methods. The hospitals that will be included in the study are illustrated in table 1.

The subjects selected will include all cleft patients born or referred to the selected hospitals from August/2010 and their age range from 0 to 12 months and their parents.

Sample group:

Study group:

Group I: Infants with non-syndromic cleft lip and/or palate

Group II: Infants with syndromic cleft lip and/or palate.

Control group:

They are normal healthy infants attending the hospitals for other reasons than clefts and their parents. The control will be matched with the study group in their age, gender, and hospital where they were located.

Method:

A formal consent form will be given to the parents after they are provided with verbal and written information concerning the importance and the procedures of the study.

Clinical examination for the Infants in the study group will be carried on in paediatric clinic using light and mirror to detect the type and characteristic of the clefts depending on the international clearinghouse for birth defects monitoring system (1991,2001)[35,36] the cleft lip and/ or palate will be defined by the examiners as:

I. Cleft lip with or without cleft palate: it is a congenital malformation characterised by partial or complete clefting of the upper lip with or without clefting of the alveolar ridge(ICBDMS 2001) [36]

    1. Isolated Unilateral Cleft lip
    2. Isolated bilateral cleft lip
    3. Unilateral Cleft lip with clefting of alveolar bone
    4. Bilateral cleft lip with clefting of alveolar bone.

II. Cleft palate without cleft lip: it is a congenital malformation characterized by a closure defect of the hard and/ or soft palate behind the foramen incisive without cleft lip. (ICBDMS 1991) [35]

    1. Complete palatal cleft
    2. Sub-mucous cleft palate
    3. Soft palate (bifid uvula).

III. Both cleft lip and palate (Shaw WC 1993) [37]:

    1. Unilateral complete cleft lip and palate.
    2. Bilateral complete cleft lip and palate.

3. Unilateral Incomplete cleft lip and palate.

4. Bilateral Incomplete cleft lip and palate.

Part II:

A questionnaire will be given to parents of the control and study groups. The co-coordinator will interview them in paediatric clinic. The average time of answering the questionnaire is 15 minutes. The questionnaire includes (see appendix A4):

- General information: date of participation, name of the hospital, place of birth, residence, day of birth, child birth order, the, name of the patients and his parents, contact numbers,

- Demographic data and pregnancy history: maternal age at delivery, maternal weight/height, length of pregnancy, duration between the last two births, prenatal visits, parents educational level, mother working status and type of occupation during pregnancy, family monthly income.

- Consanguinity and family history of birth defects: parents, grandparents, parent's sibling, 1st degree cousins, 2nd degree cousins, tribe.

- Maternal exposure to chemical and drugs during pregnancy: measure prenatal primary and secondary exposure to nicotine, glycol ether, prenatal nutrition including vitamins and folic acid. Drugs exposure; including hormones and adrenaline, stress,

Saliva sample:

Saliva sample will be collected from infants and parents from both groups. For parents, OG-500 Oragene kit will be used. Adult will be asked to spit 2ml of saliva in collecting tube to obtain 110µg DNA

For infants, OG-575 Oragene Kit will be used. The kit contains a sponge that collects saliva and then squeezed in an Oragene tube collecting tube. The saliva will be sent to the genetic lab for DNA extraction and genotyping using. It will be analysed using candidate gene approach with polymerase chain reaction (PCR).

The association of various genetic polymorphisms with the different types of clefts will be analysed on a number of candidate genes to reflect state of the science/ best evidence at the time of DNA analysis. Due to GWAS and on-going research on the genetic aetiology of cleft lip and/or palate, the choice of candidate gene will be updated shortly before the analysis. However, it is expected, depending on recent publications that the candidate genes to be studied will include:

- IRF 6
- FGFR2

Information on environmental risk factors will be correlated, compared and analysed with the DNA analysis results to examine gene-gene and gene-environmental interactions.

On-going plane:

Data collection will continue from the three cities in Saudi Arabia; Jeddah, Madinah and Riyadh. It is expected to continue for at least 6 months more to reach the

target sample size which is 150 infant-parents triad. The same number or more of matched control will be collected during the second year of this project for comparison analysis.

Table 1: Name of selected hospital in each geographic area from every city

City	Central	North	South	West	East
Riyadh	King Faisal Specialised hospital and research centre	King Fahad Medical City	King Saud Medical City	King Fahad Armed forces Hospital	King Abdulaziz Medical City
Jeddah	Maternity and children hospital, Almusaadiah and King Fahad Hospital	King Abdulaziz University Hospital	King Abdulaziz Medical City	King Fahad Armed Forces Hospital	Maternity and children Hospita, Al-Aziziah.
Madinah	Maternity and Children Hospital				

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A4: Ethical approval and consent form

Dear Parents,

I am a Paediatric Dentist from King Abdulaziz University. I and my research group are doing a study on cleft lip and palate. I am going to give you information and invite you to be part of this research. You do not need to decide today whether or not you will participate. Before you decide, you can talk to anyone you feel comfortable with about the research.

There may be some words that you do not understand. Please ask me to stop as we go through the information and I will take time to explain. If you have questions later, you can ask them to me, the study doctor or the staff.

What is cleft lip and palate?

Cleft lip and palate is a defect in the growth of the upper jaw and lip of the baby. Affected children suffer from difficult to eat and swallow, food goes back to their nose and they have hearing and speech difficulties. They also have an appearance problem.

Why do some babies have cleft lip and palate and some not?

The cause of cleft lip and palate is still unclear. It could be caused by certain medication, food or life style that affected the mother of the child during her pregnancy. Specific gene could be also the cause or both together. When there is a cause it means it could be prevented.

Why are we doing this study?

Through this study we will try to find out the cause of cleft lip and palate in Saudi Arabia, in order to control and prevent in the future. This means that you could help in protecting and saving other children from having this disease.

How can you help?

The study will need to ask you some questions regarding your pregnancy, medication, lifestyle, height/weight and occupational exposure. We will also need to

ask you information regarding family medical history, the child medical history. The questionnaire will be a personal interview and will take about 20 min. We will also need to take from your baby, mother and father a sample of blood one time only and an amount less than a tea spoon. If you do not prefer blood sample to be taken from you or your baby, you will be asked to spit saliva in a small container. However, blood is more convenient for us.

Who can help?

The persons who could participate in this study are babies born after September of 2010 and their parents. Their age should not exceed 6 months. They could have or do not have cleft lip or palate.

What will happen to the information and sample we will take?

1. Blood sample or saliva taken to the genetic lab.
2. Cells will be taken out from the sample.
3. DNA will be seen and analysed.
4. The DNA of cleft children and their parents will be compared with the DNA with non-cleft patients and their parents.
5. Information about your pregnancy and life style will be compared with the genes to find any relation.
6. If there is any relation between the gene and medication or life style we will take action to prevent cleft in the future.

The confidentiality of the research:

This information will be completely confidential. No one will be allowed to see or use it for other purpose. For the blood and saliva sample it will be stored and analysed for this research in a two years period. Any leftover blood will be destroyed or used in other researches under your permeation.

Freedom of participation:

Your participation in this research is entirely voluntary. It is your choice whether to participate or not. Whether you choose to participate or not, all the services you receive at this clinic will continue and nothing will change. If you choose not to participate in this research project, you will offered the treatment that is routinely offered in this cleft clinic in King Abdulaziz university hospital, and we will tell you more about it later. You may change your mind later and stop participating even if you agreed earlier

The duration of the research:

The procedure of the research will take about two years until the end of 2012.

Prick

Thank you for your help and time…..

Yours sincerely,

Note:

It is your right to take money to pay for your travel to the clinic/parking and we will give you [50RS] for lost work time. You will not be given any other money or gifts to take part in this research.

Question to elucidate understanding

Dear parents,

Please answer these questions before participating in the research:

- Do you know why we are asking you to take part in this study?
  ☐Yes ☐ No
- Do you know what the study is about?
  ☐Yes ☐ No
- If you decide not to take part in this research study? Do you know what your options are?
  ☐Yes ☐ No
- Do you know that you do not have to take part in this research study if you do not wish to?, ☐Yes ☐ No
- Do you know that you can withdraw anytime?
  ☐Yes ☐ No
- Can you tell me if you have understood correctly the benefits that you will have if you take part in the study?
  ☐Yes ☐ No
- Do you know if the study will pay for your travel costs and time lost, and do you know how much you will be re-imbursed?
  ☐Yes ☐ No
- Do you have any questions?
  ☐Yes ☐ No

Name of the parents:_____ Date:_____

Signature:_____

Name of the hospital: Code #:

Contact person Date:

Team contact details

Table1: members of the cleft and palate collaborating team

Speciality	Name	Contact number	email
Maxillofacial surgeon			
Paediatrician			
Plastic Surgeon			
ENT			
Paediatric dentist			
Gynaecologist			
Orthodontics			
Collaborating personal			

A5: Questionnaire

Date: _____/_____/_____ **Saudicleft project**

Questionnaire includes:

- **Section 1: Information section**

  i. Family information *

  ii. Pregnancy information

  iii. Environmental information

- **Section 2: Child examination section**

**Mother's information includes 3 months pregestation and 3 months after postgestation).*

Child's name	
File number	
Home Address	
Region	
Telephone number	
Father's mobile number	
Mother's mobile number	
Parent's email	

Section 1: Family information:

Father's information			
1. Work and work address			
2. Family income/month	[1]□Less than 4000 RS [2]□4000-7000 RS [3]□7001-10000 RS [4]□10001-16000 RS [5]□16001-23000 RS [6]□23001 or more		
3. Date of birth		**Age**	
4. Education level	[1]□No [2]□Primary [3]□Intermediate [4]□High School [5]□Bachelor [6]□Postgraduate [7]□Others_____		

Child's information			
5. Date of birth		**Age**	
6. Place of birth: Country		**City**	
7. Hospital where child born			
8. Nationality		**Sex**	[1]□Male [2]□Female
9. Gestation	[1]☐25-28w [2]☐29-32w [3]☐33 -36w [4]☐37-42 weeks		
10. Neonatal weight	[1]□1-1.5kg [2]□1.5-2kg [3]□2.1-2.5kg [4]□2.6-3kg [5]□3.1 3.5kg [6]□3.6-4kg [7]□4.1-4.5kg		
11. Head circumference	[1]☐ <30cm [2]☐32.1-34cm [3]☐34.1-36cm [4]☐36.1-38cm [5]☐38.1-40cm		
12. Length	[1]☐<38cm [2]☐38-42cm [3]☐43-47cm [4]☐48-52cm [5]☐53-57cm [6]☐More than 57cm		
13. Multiple birth?	[1]□Yes (what type?) [1]□Twins [2]□Triplets [3]□Quadruplets [2]□No (go to Q15)		
14. Type of multiple birth	[1]□All affected [2]□Same sex [3]□Different sex		

Mother's information			
15. Name		**Nationality**	
16. Residency 3 months before and after pregnancy: Country		**City**	
17. Type of area in which you lived	[1]☐Rural [2]☐Metropolis/Urban [3]☐Urban [4] ☐Industrial		
18. Date of birth		**Age**	
19. Educational level	[1]□No [2]□Primary [3]□Intermediate [4]□High school [5]□Bachelor [6]□Postgraduate		
20. Work in pregnancy and work address			
21. Height	[1]☐ <140cm [2]□141-150cm [3]□151-160cm [4]□161-170cm [5]□>171cm		
22. Weight	[1]□<40Kg [2]□41-50Kg [3]□51-60Kg [4]□61-70Kg [5]□71-80Kg [6]□81-90Kg [7]□91-100Kg [8]□101 -110Kg [9]□111Kg or more		
23. How many children do you have?	(*Circle the correct answer*): 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15		
24. What is the birth order of the child?	(*Circle the correct answer*): 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15		
25 What is the duration between this child and the one before in years?	[1]☐1 or less [2]☐1.1-2Y [3]☐2.1-3Y [4]☐3.1-4Y [5]☐4.1-5Y [6]☐5.1-6 [7]☐6.1-7 [6]☐more than 7		
26. Did you have any miscarriages? How many?	*(Circle the correct number)*: 0 1 2 3 4 5 6		

Section 1: Pregnancy information:

29. How many times did you visit your Dr. 3 months before your pregnancy? *Circle the number*: 0 1 2 3

30. How many times did you visit your Dr. in the first trimester? *Circle the correct number*: 0 1 2 3 4 5 6

31. Was the pregnancy planned? [1]□Yes [2]□No

30. Did you have medication, X-rays or Ultrasounds? [1]☐Yes (*please answer from 31-42*) [2]☐No (*go to Q43*)

Please record the type and duration of the drugs you used:

	Before pregnancy				**In the 1st trimester**			
Medication	**Dose**	**#/day**	**#of days**	**Brand**	**Dose in mg**	**#/day**	**#of days**	**Brand**
31. Antibiotics Name:_____	[1]☐250 [2]☐500 [3]☐1000	1 2 3 4	[1]□1-4 [2]□5-8 [3]□9-12 [4]□>12 days		[1]☐250 [2]☐500 [3]☐1000	1 2 3 4	[1]☐1-4 [2]☐5-8 [3]☐9-12 [4]☐>12	
32. Folic Acid	[1]□4 [2]□5 [3]□With multivitamin	1 2 3 4	[1]☐<30 [2]☐31-60 [3]☐61-90		[1]□4 [2]□5 [3]□With multivitamin	1 2 3 4	[1]☐<30 [2]☐31-60 [3]☐61-90	
33. Multivitamins								
34. Iron								
35. Sickness Drug								
36. Cortisone								
37. Anticonvulsant								
38. Insulin								
39. Contraceptive								
40. Other, please list **a. paracetamol:** **b.**								
	# of times		**When?**		**# of times**		**When?**	
41. X-Ray, CT, etc	0 1 2 3 4				0 1 2 3 4			
42. Ultrasound	0 1 2 3 4				0 1 2 3 4			

43. Did you suffer from any illnesses during your pregnancy? [1]□Yes (answer 44-59) [2]□No (go to Q60)

Type of disease	Before pregnancy		1st trimester	
	Severity	# of days	Severity	# of days
44. Viral infection	[1]□Mild [2]□Mod [3]□Severe	[1]□1-3 [2]□4-6 [3]□>7	[1]□Mild [2]□Mod [3]□Severe	[1]□1-3 [2]□4-6 [3]□>7
45. Flu	[1]□Mild [2]□Mod [3]□Severe	[1]□1-3 [2]□4-6 [3]□>7	[1]□Mild [2]□Mod [3]□Severe	[1]□1-3 [2]□4-6 [3]□>7
46. Fever	[1]□Mild [2]□Mod [3]□Severe	[1]□1-3 [2]□4-6 [3]□>7	[1]□Mild [2]□Mod [3]□Severe	[1]□1-3 [2]□4-6 [3]□>7
47. High blood pressure	[1]□Mild [3]□Severe	[1]□1-5 [2]□6-10 [3]□11-15 [4]□ >15	[1]□Mild [3]□Severe	[1]□1-5 [2]□6-10 [3]□11-15 [4]□ >15
48. Diabetes	[1]□Mild [2]□Mod [3]□Severe	□<14 [2]□15-30 [3]□>30	[1]□Mild [2]□Mod [3]□Severe	[1]□<14 [2]□15-30 [3]□>30
49. Depression	[1]□Mild [2]□Mod [3]□Severe	[1]□<14 [2]□15-30 [3]□>30	[1]□Mild [2]□Mod [3]□Severe	[1]□<14 [2]□15-30 [3]□>30
50. Convulsion	[1]□Mild [2]□Mod [3]□Severe	[1]□1-3 [2]□4-6 [3]□>7	[1]□Mild [2]□Mod [3]□Severe	[1]□1-3 [2]□4-6 [3]□>7
51. Renal disease	[1]□Mild [2]□Mod [3]□Severe	[1]□<14 [2]□15-30 [3]□>30	[1]□Mild [2]□Mod [3]□Severe	[1]□1-3 [2]□4-6 [3]□>7
52. Liver Disease	[1]□Mild [2]□Mod [3]□Severe	□<14 [2]□15-30 [3]□>30	[1]□Mild [2]□Mod [3]□Severe	[1]□<30 [2]□31-60 [3]□>90
53. Vaginal Bleeding	[1]□Mild [2]□Mod [3]□Severe	[1]□1-3 [2]□4-6 [3]□>7	[1]□Mild [2]□Mod [3]□Severe	[1]□1-3 [2]□4-6 [3]□>7
54. Chronic Disease	[1]□Mild [2]□Mod [3]□Severe	[1]□<14 [2]□15-30 [3]□>30	[1]□Mild [2]□Mod [3]□Severe	[1]□<30 [2]□31-60 [3]□>90
55. Asthma	[1]□Mild [2]□Mod [3]□Severe	[1]□1-3 [2]□4-6 [3]□>7	[1]□Mild [2]□Mod [3]□Severe	[1]□1-3 [2]□4-6 [3]□>7
56. Abdominal pain	[1]□Mild [2]□Mod [3]□Severe	[1]□<14 [2]□15-30 [3]□>30	[1]□Mild [2]□Mod [3]□Severe	[1]□<14 [2]□15-30 [3]□>30
57. Severe morning sickness	[1]□ 1st month [2]□2nd month [3]□3rd month [4]□all		[1]□1-10 day [2]□11-20day [3]□21-30	
58. Threatened Abortion	[1]□ 1st month [2]□2nd month [3]□3rd month [5]□all		[1]□1-10day [2]□11-20day [3]□21-30	
59. Other, please list **a. e.g. Migraine** **b.** **c.**	[1]□Mild [2]□Mod [3]□Severe	[1]□<14 [2]□15-30 [3]□>30	[1]□Mild [2]□Mod [3]□Severe	[1]□<14 [2]□15-30 [3]□>30

60. Were you exposed to any chemicals in your home/work environment? [1]□Yes (answer 61-67) [2]□No (go to 68)

Type of chemical	Before pregnancy		1st trimester	
	Amount	# days	Amount	# days
61. Cyanides		[1]□1-3 [2]□4-6 [3]□>7 days		[1]□1-3 [2]□4-6 [3]□>7 days
62. Pesticides	[1]□ 1 room [2]□ >1room	[1]□1-3 [2]□4-6 [3]□>7 days		[1]□1-3 [2]□4-6 [3]□>7 days
63. Solvents (*thinner Acetone)*		[1]□1-3 [2]□4-6 [3]□>7 days		[1]□1-3 [2]□4-6 [3]□>7 days
64. Pollutant	[1]□ 1 room [2]□ >1room	[1]□<30 [2]□31-60 [3]□>90		[1]□<30 [2]□31-60 [3]□>90

65. Computer, copying machine (<2m away)	# of hours/day	[1]☐<30 [2]☐31-60 [3]☐>90	# of hours/day	[1]☐<30 [2]☐31-60 [3]☐>90
66. Microwaves (mobile, cooking, others)	# of hours/day	[1]☐<30 [2]☐31-60 [3]☐>90	# of hours/day	[1]☐<30 [2]☐31-60 [3]☐>90
67. Other, please list				

Section 1 Environmental information

68. Do you smoke? [1]☐Yes (answer Q69 to Q72) [2]☐No (go to Q73)

Type of Smoking	**Before pregnancy #**			**1st trimester #**		
	# /day	**#days /week**	**#of weeks**	**# /day**	**# days /week**	**#of weeks**
69. Smoking tobacco		0 1 2 3 4 5 6 7			0 1 2 3 4 5 6 7	
70. Non smoking tobacco		0 1 2 3 4 5 6 7			0 1 2 3 4 5 6 7	
71. Sheesha (Jorak)	0 1 2 3	0 1 2 3 4 5 6 7		0 1 2 3	0 1 2 3 4 5 6 7	
72. Sheesha (Moasel)	0 1 2 3	0 1 2 3 4 5 6 7		0 1 2 3	0 1 2 3 4 5 6 7	
Tambak (used in Yaman)						

73. Does the father of the child smoke? [1]☐Yes [2]☐No (go to Q78)

Type of Smoking	**Before pregnancy #**			**1st trimester #**		
	# /day	**#days /week**	**#of weeks**	**# /day**	**# days /week**	**#of weeks**
74. Smoking tobacco		0 1 2 3 4 5 6 7			0 1 2 3 4 5 6 7	
75. Non smoking tobacco		0 1 2 3 4 5 6 7			0 1 2 3 4 5 6 7	
76. Sheesha(Jorak)	0 1 2 3	0 1 2 3 4 5 6 7		0 1 2 3	0 1 2 3 4 5 6 7	
77. Sheesha (Moasel)	0 1 2 3	0 1 2 3 4 5 6 7		0 1 2 3	0 1 2 3 4 5 6 7	

78. How many cigarettes are smoked around you at home or work? Hours/day_____ days/ week _____

79. Have you suffered from any family problems during your pregnancy? [1]☐Yes [2]☐No

80. Do you think you were under any pressure during your pregnancy? [1]☐Yes [2]☐No

81. What sort of water do you drink [1]☐Tap [2]☐Bottled [3]☐Well [4]☐Others_____

82. For drinking bottled water, what is the name of the company? (*look at the Show card):*_____

83. How many days do you eat food containing flour per week? (Circle the # of days): 0 1 2 3 4 5 6 7

84. How many days you cook with flour per week? (Circle the # of days): 0 1 2 3 4 5 6 7

85. What kind of flour do you use? _____

86. Do any of your relatives have birth defects? [1]☐Yes (answer 87-95) [2]☐No(go to Q96) [3]☐Do not know (go to Q96)

If yes, please specify the relation to the child and type of defect:

Relation to the child	Defect Type *	Date of birth or age
87.	88.	89
90.	91.	92.
93.	92.	95.

*[1]☐cleft lip and palate [2]☐only cleft palate [3]☐limb abnormality [4]☐CV [5]☐other orofacial defects [6]☐genitourea [7]☐multiple defects [8]☐others_____

96. Are the parents related (Consanguinity)? [1]☐Yes (answer Q96) [2]☐No(go to Q97)

97. What is the relation? [1]☐1st degree cousin [2]☐1st cousin once removed [3]☐2nd degree cousin [4]☐Same tribe. [5]☐double 1st degree cousin

Section 2: Child examination

98. Have photos been taken: [1]☐Yes (Frontal, lateral, occlusal) [2]☐No

99. Was the cleft prenatally diagnosed? [1]□Yes [2]□No

100. Any associated anomalies with cleft lip and palate? [1]☐Yes (answer Q100) [2]☐No (go to Q101)

101. Describe the associated anomaly:

_____

[1]☐Congenital heart disease [2]☐Limbs malformation [3]☐Polydactyly [4]☐Hydrocephaly [5]☐UT defects

[6]☐Other facial anomaly: _____ [7]☐Others: _____

Type of cleft:

102. Is the cleft:

[1]☐Syndromic Name or code of the Syndrome: _____

[2]☐Non-syndromic. [3]☐Multi-malformed infant [4]☐Part of Pierre Robin sequence

Cleft description:

Name of category	**Right**	**Left**
Simonart band	103. [1]□Yes [2]□No	104.[1]□Yes [2]□No
Cleft lip	105.[1]□Yes [2]□No	106.[1]□Yes [2]□No
Alveolus	107. [1]□Yes [2]□No	108.[1]□Yes [2]□No
Hard palate	109.[1]□Yes [2]□No	
Soft palate	110.[1]□Yes [2]□No	

Thank you for making a difference in another child's life!!

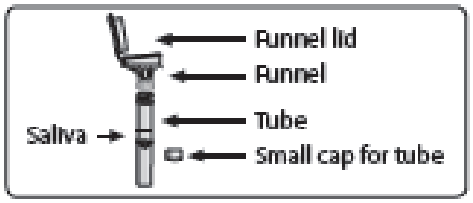
A6: Technique for collecting Saliva from adult using 500 Oragene kit

ORAgene·DNA

REF OG-500/510

USER INSTRUCTIONS

Read all instructions prior to collection



Collection precautions:

Do NOT eat, drink, smoke or chew gum for 30 minutes before giving your saliva sample.

Do NOT remove the plastic film from the funnel lid.

Intended use: For the collection of human DNA from saliva samples.

Contents: Kit contains stabilizing liquid.

Warnings and precautions: Wash with water if stabilizing liquid comes in contact with eyes or skin. Do NOT ingest. See MSDS at www.dnagenotek.com.

Small cap, choking hazard.

Storage: 15°C./ 30°C

Summary and explanation of the kit:

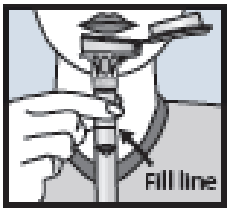
Oragene•DNA is a self-collection kit that provides the materials and instructions for collecting and stabilizing saliva specimens.

Label legend:

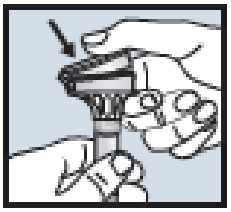
- Consult package insert
- Collect saliva by (Use by)
- In vitro diagnostic medical device
- REF Catalog number
- CE CE Marking
- Caution, consult instructions for use
- STORE Storage instructions
- Authorized Representative
- Manufacturer

Procedure:

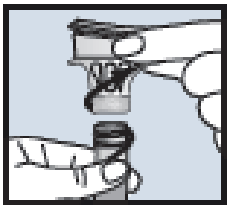
Most people take between 2 and 5 minutes to deliver a saliva sample following steps 1 to 5.



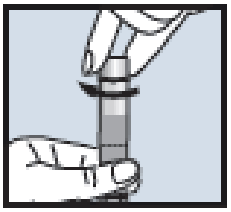
1 Spit into funnel until the amount of liquid saliva (not bubbles) reaches the fill line shown in picture #1.



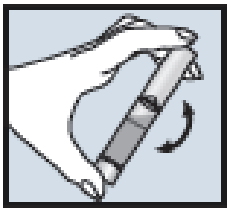
2 Hold the tube upright with one hand. Close the funnel lid with the other hand (as shown) by firmly pushing the lid until you hear a loud click. The liquid in the lid will be released into the tube to mix with the saliva. Make sure that the lid is closed tightly.



3 Hold the tube upright. Unscrew the funnel from the tube.



4 Use the small cap to close the tube tightly.



5 Shake the capped tube for 5 seconds. Discard or recycle the funnel.

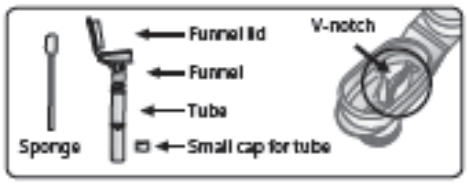
A7: Technique for collecting Saliva from infants using 575 Oragene kit.

ORAgene·DNA

REF OG-575

USER INSTRUCTIONS

Read all instructions prior to collection



Collection precautions:

Do NOT remove the plastic film from the funnel lid.

Check sponge for damage each time before inserting into donor's mouth. Use second sponge if first sponge shows any signs of wear or tear.

Do NOT substitute with other sponges or swabs.

Intended use: For the assisted collection of human DNA from saliva samples.

Contents: Kit contains stabilizing liquid.

Warnings and precautions:

Choking hazards:
- Small cap in collection kit.
- Plastic bag containing sponges.
- Caution should be used when inserting sponge into donor's mouth.

For supervised collections:
- Do NOT leave donor unattended.
- Do NOT allow donor to handle the sponge, small cap or packaging.
- Wash with water if stabilizing liquid comes in contact with eyes or skin. Do not ingest. See MSDS at www.dnagenotek.com.

Storage: 15°C – 30°C

Summary and explanation of the kit:

Oragene·DNA is an assisted collection kit that provides the materials and instructions for collecting and stabilizing saliva specimens.

Label legend:

	Consult package insert
	Collect saliva by (Use by)
	In vitro diagnostic medical device
REF	Catalog number
CE	CE Marking
	Caution, consult instructions for use
15°C – 30°C	Storage instructions
	Authorized Representative
	Manufacturer

Procedure:

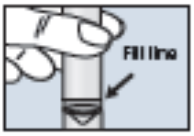
Ensure donor does NOT eat, drink, smoke or chew gum for 30 minutes before collecting a saliva sample.

Ensure donor is in an upright position during sample collection.

It may take up to 15 minutes to collect a saliva sample following steps 1 to 7.

1 Place one sponge in cheek pouch. Gently move the sponge along the gums and inner cheeks for 30 seconds to soak up as much saliva as possible.

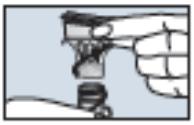
2 Once sponge is saturated with saliva, insert sponge in V-notch of funnel. Wring saliva out of sponge using a twisting and pushing motion against the inner wall of the V-notch. Saliva will flow into tube.



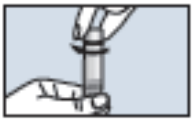
3 Repeat these steps (1 to 2) USING THE SAME SPONGE until the liquid saliva (not bubbles) reaches the fill line. Check sponge for damage each time before inserting into donor's mouth. Use second sponge if first sponge shows any signs of wear or tear.

Tap tube bottom against hard surface to reduce number of bubbles.

4 Hold the tube upright with one hand. Close the lid with the other hand (as shown) by firmly pushing the lid until you hear a loud click. The liquid in the lid will be released into the tube to mix with the saliva. Make sure the lid is closed tightly.



5 Hold the tube upright. Unscrew the funnel from the tube.



6 Use the small cap to close the tube tightly.

7 Shake the capped tube for 5 seconds. Discard the funnel and sponges.

A8: Arabic consent form and in questionnaire

A8.1: Arabic consent form

دراسة أسباب حدوث الشفة الأرنبية وشق سقف الحلق

عزيزي المواطن..

سيشرح لك عضو من فريق البحث محتويات هذه الدراسة وتأثيرها عليك .و يصف هذا الإقرار إجراءات الدراسة ، والمخاطر والفوائد من المشاركة ، وكيفية الحفاظ على سرية المعلومات .الرجاء اخذ الوقت الكافي في طرح الأسئلة لكي تتخذ قرارك ما إذا كنت ستشارك أم لا .وهذه الموافقة تسمى الموافقة المستنيرة .إذا قررت المشاركة في هذه الدراسة ، سيطلب منك التوقيع على هذا الإقرار وستعطي نسخة لسجلاتك .وطوال هذا الإقرار اللفظ،" أنت "سوف يشير إليك أو إلى طفلك ، حسب الاقتضاء .

لماذا تجري هذه الدراسة؟

للحد من إنتشار و حدوث الشفة الأرنبيه و شق سقف الحلق في المستقبل

وكم عدد المشاركين في هذه الدراسة ؟

300طفل ووالديهم

ماذا سيحدث إذا شاركت في هذه الدراسة ؟

ستشارك في حماية الأطفال الذين سيولدون في المستقبل من أن يحدث لهم خلل في تكوين الحلق أو الشفة

ما هو متوقع من خلال دراسة لي؟

سنأخذ منك بعض المعلومات العامة عنك وعن بعض الأمور و الأدوية والأحداث المتعلقة بالحمل .كما لأننا سنحتاج إلى عينة دم إن أمكن في بعض الحالات أو فقط عينة لعابية أن كنت لا تريد أن تعطي عينة دم

.ما هي مدة المشاركة في هذه الدراسة؟

منك لا نحتاج إلا زيارة واحده .أما الدراسه نفسها فستستمر لمدة 1 إلى 2 سنوات

هل أستطيع إنهاء المشاركة ؟

نعم .يمكنك أن تقرر التوقف في أي وقت .فقط اخبر الطبيب إذا قررت التوقف .ليوضح لك كيفية إنهاء مشاركتك بأمان .لا أحد سيحملك علي تغيير رأيك

هل هناك مخاطر متوقعة إذا أنهيت المشاركة في الدراسة ؟

لا ,لا يوجد أي مخاطر

ما هي المخاطر أو الآثار الجانبية التي يمكن حدوثها من جراء المشاركة في الدراسة؟

لا يوجد إلا غزة الإبرة إن وافقت على أخذ عينة دم

هل هناك فوائد من المشاركة في الدراسة ؟

نعم ,هناك فائده لغيرك من الأطفال الذين سيولدون فبي المستقبل

مشاركتك في هذه الدراسة قد لا تؤدي إلي تحسن حالتك.ولكن يأمل الأطباء أن يكون الاجراء مفيد لغيرك عن طريق فهمن أكثر للمرض و أسبابه ولا يوجد دليل على ذلك حتى الآن

وما هي تكاليف المشاركة في الدراسة ؟

لن تتحمل تكاليف أي من أنشطة الدراسة .

هل سأتقاضى اجر نظير المشاركة في هذه الدراسة ?

في مقابل وقتك، جهدك ونفقات السفر سيدفع لك50 ، للمشاركة في هذه الدراسة .

هل سيتم الحفاظ علي المعلومات الطبية الخاصة بي بسرية ؟

سنبذل قصارى جهدنا للتأكد من أن المعلومات الشخصية في سجلك الطبي تحظى بالسرية .ومع ذلك ، لا يمكننا أن نضمن الخصوصية التامة .يمكن أن يفصح عن معلوماتك الشخصية إذا اقتضى الأمر وذلك بموجب القانون .لن يتم الإفصاح عن اسمك أو المعلومات الشخصية إذا تم نشر نتائج هذه الدراسة نشرت أو عرضت في الاجتماعات العلمية

قرار المشاركة في هذه الدراسة من اختيارك .لك حرية اختيار المشاركة في هذه الدراسة أو لا. كما يمكنك إنهاء المشاركة في أي وقت .مهما كان قرارك ، لن يكون هناك أي عقوبة و لن تفقد أي من الفوائد العادية الخاصة بك .ترك الدراسة لن يؤثر علي الرعاية الطبية المقدمة لك .د .هبه قد يستخدم المعلومات التي تم جمعها قبل أن تترك لدراسة.

ونحن سوف نبلغك بكل المعلومات والمستجدات أو التغييرات في الدراسة التي يمكن أن تؤثر على صحتك أو على استعدادك لمواصلة الدراسة.

هل يمكنني أن أحصل على نتائج البحث؟

نعم ,يمكننا إبلاغك بالنتائج إن أحببت بعد الإنتهاء منها خلال سنتين.

نشكر لكم تعاونكم معنا وحرصكم على أن تكونوا جزأ من هذه الرسالة من أجل مستقبل أفضل لأبنائنا.....

التوقيع:

A8.2: Arabic questionnaire

بحث عن أسباب حدوث الشفة الأرنبية وشق سقف الحلق في المملكه العربية السعودية

معلومات عن الطفل

1. الأسم_____ _____ _____ _____
2. عنوان المنزل :حي_____شارع_____مدينة_____ص ب_____رمز بريدي_____
3. رقم الهاتف _____ جوال الأب _____جوال الأم_____
4. عمل الأب_____:عمل الأم_____:
5. عنوان عمل الأب_____:
6. عنوان عمل الأم_____:
7. الدخل الشهري للأسرة □ أقل من 4000 رس □ 4000-6000 □ 7000- 9000□ 10000- 15000□ 10000-14000□ 21000-30000□ 6000-20000□ أكثر من30000
8. عمر الأب :
9. الجنسية_____
10. الجنس :أنثى□ ذكر□
11. تاريخ الولادة:_____
12. مكان الولادة :اسم المستشفى _____ :المدينة_____المنطقة _____
13. وزنه عند الولادة_____ كجم
14. محيط مدار الرأس_____
15. طوله عند الولادة_____

_____

معلومات عن الأم

16. الاسم
17. عمر الأم
18. الجنسية_____
19. مكان إقامتها أثناء الحمل_____
20. المدينة_____ المنطقة_____
21. لطول: □ أقل من140سم 141-150سم□ 151-160□ 161-170□-
22. الوزن قبل الحمل مباشرة:أقل من 40سم□ 41-50□ 51-60□ 61-70□ 71-80□ 81-90□ 91-100□ أكثر من 100□
23. مستوى التعليم

غير متعلم□ ابتدائي□ متوسط□ ثانوي□ جامعي □

24. المهنة_____:

25. ضع دائرة على الأبناء للأم 14 13 12 11 10 9 8 7 6 5 4 3 2 1 :

26. ضع دائرة على ترتيب هذا الطفل بين إخوانه؟ 10 9 8 7 6 5 4 3 2 1

27. ما هو الفترة الزمنية بين حملك بهذا الطفل ومن قبله : □أقل من 1 □ 1-2 □2-3 □3-4 □5-6 □أكثر من 6

28. هل كان هنا إسلابات؟ نعم□ لا□

إذا كانت الإجابة بنعم.....

29. .ضع دائرة على عددها 6 5 4 3 2 1 :

30. هل كان حملك بهذا الطفل مخطط له؟ نعم□ □لا

31. كم كان عدد زيارتك الأولى لطبيب النساء و الولاده قبل الحمل؟ 1 2 3

32. كم كان عدد زيارتك الأولى لطبيب النساء و الولاده في 3أشهر الأولى للحمل؟ 1 2 3

33. هل تستعملين الدخان؟ نعم□ لا

إذا كانت الإجابة بنعم.....

34. ما نوعه:

أشهر الأولى	قبل الحمل	نوع التدخين
		36. سجائر
		37. شيشة
		38. أخرى

39. هل الأب مدخن؟ نعم□ لا□

40. ما نوعه:

أشهر الأولى	قبل الحمل	نوع التدخين
		42. سجائر
		43. شيشة
		44. أخرى

45. هل أحد من الأقارب الذين تحتكين بهم كثيرا يدخن (أخ ,أب ,ابن)؟ نعم□ لا□

46. هل أخذت أدوية أثناء الحمل :نعم □ لا□

3الأشهر الأولى	قبل الحمل	النوع
		47. فيتامينات
		50. حديد
		53. فولك أس

		56. انسولين
		59. مضاد حيوي
		62. أدوية للغثيان
		65.
		68.

71. هل أصبت بأي مرض أثناء الحمل؟

72. □نعم□ لا

3أشهر الأولى	قبل الحمل	النوع
		73. زكام
		76. التهاب فيروسي
		79. سخونة
		82. الغثيان
		85. ضغط
		88. سكر
		91.

94. ما نوع الماء الذي تشربينه؟ زمزم□ صحة□ أبار □ تحلية□ أخرى□ _____غير معروف

95. هل تعرضت لمواد مضرة أثناء الحمل: نعم□ لا□

أشهر الأولى	ل الحمل	وع
		يدات حشريه
		مذيبات
		بخور
		جوالات
		كمبيوتر

114. هل كان هناك ضغوط غير عاديه عليك أثناء الحمل؟ نعم□ لا□

115. هل كان هناك مشاكل عائلية أثناء الحمل؟ نعم□ لا□
116. هل هناك شخص أخر في العائلة مصاب بالشفة الأرنبية أو شق سقف الحلق؟ نعم لا

إذا كانت الإجابة بنعم.....

117. مانوع الشق□ :بِشفة الأرنبية□ شق سقف الحلق□ الاثنين □ لا نعلم
118. من في العائلة؟

الأم□ الأب□ الجد من الأم □ الجدة من الأم□ الجد من الأب□ الجدة من الأب □
الأعمام□ الأخوال□ أبناء العم□ أبناء الخال□ أخ□ أخت
□أخرى_____

119. هل هناك شخص أخر في العائلة مصاب بأي نوع أخر من التشوهات الخلقية؟ نعم□ لا
120. إذا كانت الإجابة بنعم.....
121. ما نوع التشوه؟_____
122. من في العائلة؟
123. الأم□ الأب□ الجد من الأم □ الجدة من الأم□ الجد من الأب□ الجدة من الأب □
الأعمام□ الأخوال□ أبناء العم□ أبناء الخال□ أخ□ أخت
□أخرى_____
124. .25هل الوالدين أقارب؟
125. □نعم□ لا
126. إذا كانت الإجابة بنعم.....
127. من في العائلة؟ □ أبناء عم□ أبناء خال أو عم الأب أو الأم
□أخرى_____

نشكركم على تعاونكم معنا ....

A9: List of questionnaire code; Hospital code and code for each question:

The Saudicleft main code	the first two digit as the hospital code
	the third digit is the group code(study or control)
	the rest is the serial number of the patient
Information:	
Father's information	
DOB	DOB in Hejry, Gregorian or just right the age. Whatever the patient remembers
age	1=less than 20, 2=21-40, 3=41-60
Work	Type of occupation: teacher, physician, engineer, military…etc
Monthly income	This include the total monthly income of the family (father's salary+ mother's salary+ any other incomes)
Education level	His last educational level he reached at school
Child's information	
DOB	da/mo/year
place of birth	name of hospital
	government+Tertiary hospital=11
	government+2ry hospital=12
	Private+Tertiary hospital=21
	Private+2ry hospital= 22
	name of the city
	name of the country
child's nationality	Saudi, middle east countries (other than Saudi), Asian countries, western countries, North Africa, other African countries.
Neonatal weight	ranging from 1 Kg to 4.5 KG
Gestation	weeks of pregnancy (from conception to delivery)
Head circumference	in CM
Neonatal length	in CM
Is the child is single or twin	0=no twins

Description of the twins	sex (1= same or 2= different)
	1= both affected or only 2= one affected
Mother's information	
Name	
Nationality	1=Saudi
	2= middle east countries (other than Saudi)
	3=Asian countries,
	4=western countries
	5=North Africa
	6= Other African countries
residency	name of the city country and region where she lived 3 months before and 3 months after her pregnancy
describe the area you live in	1= Rural: if she lived in a community with a population less than one hundred thousand
	2=Metropolis/urban: if she lived in a big city with population greater than one million
	3= Urban: if she lived in a population of about 500,000
	4= Industrial: if she is living in a zone that is less than 10Km from industries and factories
Work	Type of occupation: teacher, physician, engineer, military…etc
Education level	Her last educational level she reached at school
region	In which of he 13 regions in Saudi Arabia you lived 3 months before and 3 months after your pregnancy
Height	In cm to measure BMI of the mother
Weight	the mother weight just before she got pregnant in Kg to measure her BMI
number of children	The total number of children the mother delivered including the cleft child and the still-berths
The order of the child between his siblings	The cleft child position among sibs (including abortions and stillbirths from the same mother)
	1= 1st
	2= 2nd
	etc...
duration between mother pregnancy with cleft child and the one before	The number of years between the delivery of the cleft child and the child before.

	1= if 1 year or less
	2= if 2 year or less but more than 1 year
	etc...
The number of miscarriages the mother had	0= no miscarriage
	1= only one miscarriage
	2= two **miscarriages**
	3= three miscarriages
	etc...
Water intake:	
The type of water the mother drank during the 3months before and after pregnancy	1= tap water
	2= bottled water
	3= Zamzam
If the mother drank bottled water, please specify the origin of the water	by writing the name of the product we can find out where the water came from
The amount of flour intake:	
How many days per week you cook with flour	0= do not cook with flour
	1= once a week
	2=twice a week
	etc...
How many days per week you eat food containing flour	like bread, cereals and other containing flour product
The name of flour product they use	To know the origin of the flour. Most of the Saudi people either use Saudi or Kuwaiti flour
	1=Saudi flour
	2=Kuwaiti flour

Pregnancy:	
Follow up with Dr:	
Before pregnancy	0=never
	1=once
	2=twice
	3=three times
In the first trimester	0=never
	1=once
	2=twice
	3=three times
Was the pregnancy planned	1=yes
	2=no
Medication used	
Antibiotics In the three months before pregnancy	1=yes: dose & duration
	2=no
Antibiotics In the three months after pregnancy	1=yes: dose & duration
	2=no
Folic acid In the three months before pregnancy	1=yes: dose & duration
	2=no
Folic Acid In the three months after pregnancy	1=yes: dose & duration
	2=no
Folic acid with multivitamins In the three months before pregnancy	1=yes: dose & duration
	2=no
Folic Acid with multivitamins In the	1=yes: dose & duration

three months after pregnancy	
	2=no
Multivitamins In the three months before pregnancy	1=yes: dose & duration
	2=no
Multivitamins In the three months after pregnancy	1=yes: dose & duration
	2=no
Iron In the three months before pregnancy	1=yes: dose & duration
	2=no
Iron In the three months after pregnancy	1=yes: dose & duration
	2=no
Sickness Drug In the three months before pregnancy	1=yes: dose & duration
	2=no
Sickness Drug In the three months before pregnancy	1=yes: dose & duration
	2=no
Cortisone In the three months after pregnancy	1=yes: dose & duration
	2=no
Cortisone In the three months before pregnancy	1=yes: dose & duration
	2=no
Anticonvulsant In the three months after pregnancy	1=yes: dose & duration
	2=no
Anticonvulsant In the three months before	1=yes: dose & duration

pregnancy	
	2=no
Insulin In the three months after pregnancy	1=yes: dose & duration
	2=no
Insulin In the three months before pregnancy	1=yes: dose & duration
	2=no
Contraceptive In the three months before pregnancy	1=yes: dose & duration
	2=no
Contraceptive In the three months after pregnancy	1=yes: dose & duration
	2=no
Exposure to X-ray 3 month before pregnancy	1=yes: number of times
	2=no
Exposure to X-ray 3 month after pregnancy	1=yes: dose & duration
	2=no
Frequency of Ultrasound examination before pregnancy	1=yes: number of times
	2=no
Frequency of Ultrasound examination 3 months after pregnancy	1=yes: number of times
	2=no
Disease	
Viral infection in the 3 month before pregnancy	Onset of the disease and duration
	0=not affected

	1= mild infection
	2= mod
	3=severe
viral infection in the 3 month after pregnancy	Onset of the disease and duration
	0=no
	1= mild
	2= mod
	3=severe
	2=no
Flu in the 3 month before pregnancy	Onset of the disease and duration
	1= mild flu
	2= mod
	3=severe
Flu in the 3 month after pregnancy	Onset of the disease and duration
	1= mild flu
	2= mod
	3=severe
Fever in the 3 month before pregnancy	Onset of the disease and duration
	0=no fever
	1= mild
	2= mod
	3=severe
Fever in the 3 month after pregnancy	Onset of the disease and duration
	0=no fever
	1= mild
	2= mod

	3=severe
High blood pressure in the 3 month before pregnancy	0=no HBP
	1=mild, when the BP range from 140/90 to 160/110
	2=sever if BP was greater than 160/110
High blood pressure in the 3 month after pregnancy	0=no HBP
	1=mild, when the BP range from 140/90 to 160/110
	2=sever if BP was greater than 160/110
Diabetes in the 3 month before pregnancy	1=yes: onset of the disease and duration
	2=no
Diabetes in the 3 month after pregnancy	1=yes: onset of the disease and duration
	2=no
49. Depression in the 3 month before pregnancy	1=yes: onset of the disease and duration
	2=no
49. Depression in the 3 month after pregnancy	1=yes: onset of the disease and duration
	2=no
50. Convulsion in the 3 month before pregnancy	1=yes: onset of the disease and duration
	2=no
50. Convulsion in the 3 month after pregnancy	1=yes: onset of the disease and duration
	2=no
51. Renal disease in the 3 month before pregnancy	1=yes: onset of the disease and duration
	2=no
51. Renal disease in the 3 month after pregnancy	1=yes: onset of the disease and duration

	2=no
52. Liver Disease in the 3 month before pregnancy	1=yes: onset of the disease and duration
	2=no
52. Liver Disease in the 3 month after pregnancy	1=yes: onset of the disease and duration
	2=no
53. Vaginal Bleeding in the 3 month before pregnancy	1=yes: onset of the disease and duration
	2=no
53. Vaginal Bleeding in the 3 month after pregnancy	1=yes: onset of the disease and duration
	2=no
54. Chronic Disease in the 3 month before pregnancy	1=yes: onset of the disease and duration
	2=no
54. Chronic Disease in the 3 month after pregnancy	1=yes: onset of the disease and duration
	2=no
55. Asthma in the 3 month before pregnancy	onset of the disease and duration
	0=no Asthma
	1= mild Asthma
	2= mod
	3=severe
55. Asthma in the 3 month after pregnancy	onset of the disease and duration
	0=no Asthma
	1= mild Asthma
	2= mod

	3=severe
56. Abdominal pain in the 3 month before pregnancy	by giving the patient a pain score from 1 to 10
	0=no pain
	1=mild, if the patient give a score less than 5
	2=moderate, if the patient give a score of 5
	3=severe, if the patient give a score more than 5
56. Abdominal pain in the 3 month after pregnancy	by giving the patient a pain score from 1 to 10
	0=no pain
	1=mild, if the patient give a score less than 5
	2=moderate, if the patient give a score of 5
	3=severe, if the patient give a score more than 5
57. Severe morning sickness in the 1st trimester	1=yes, if the number of vomiting is more than 6, then mention the duration
	2=no
58. Threatened Abortion in the first trimester	1=yes: onset of the disease and duration
	2=no
Exposed to chemicals	
Cyanides 3 months before pregnancy	0=not exposed
	1= exposed 1-3 days
	2=exposed 4-6 days
	3 exposed more than 7 days
Cyanides exposure in the 1st trimester	0=not exposed
	1= exposed 1-3 days

	2=exposed 4-6 days
	3 exposed more than 7 days
Pesticides exposure 3 months before pregnancy	0=not exposed
	1= exposed 1-3 days
	2=exposed 4-6 days
	3 exposed more than 7 days
Pesticides exposure in the 1st trimester	0=not exposed
	1= exposed 1-3 days
	2=exposed 4-6 days
	amount: 1=one room (low amount) , 2=all the house(large amount)
	3 exposed more than 7 days
Pollutant exposure 3 months before pregnancy	0=not exposed
	1= exposed 1-3 days
	2=exposed 4-6 days
	3 exposed more than 7 days
Pollutant exposure in the 1st trimester	0=not exposed
	1= exposed 1-3 days
	2=exposed 4-6 days
	3 exposed more than 7 days
Computer exposure in the 3 months before pregnancy	multiply the numbers of hours per day with the days to get the total hours exposure in the 3 months before pregnancy
	0=not exposed
	1= exposed <30 hours
	2=exposed 30-60 hours
	3= exposed more than 60 hours
Computer exposure in the	multiply the numbers of hours per day with the days to get the total hours exposure in the 3 months before

1st trimester	pregnancy
	0=not exposed
	1= exposed <30 hours
	2=exposed 30-60 hours
	3= exposed more than 60 hours
Microwaves exposure in the 3 months before pregnancy	multiply the numbers of hours per day with the days to get the total hours exposure in the 3 months before pregnancy
	0=not exposed
	1= exposed <30 hours
	2=exposed 30-60 hours
	3= exposed more than 60 hours
Microwaves exposure in the 1st trimester	multiply the numbers of hours per day with the days to get the total hours exposure in the 3 months before pregnancy
	0=not exposed
	1= exposed <30 hours
	2=exposed 30-60 hours
	3= exposed more than 60 hours
Smoking	
Did the mother smoke smoking tobacco 3 months before pregnancy	multiply the number of cigarette with the number of days in the three months to get the total cigarette used per 3months
	0=did not
	1=30 cigarette /3 month before pregnancy
	2=31-60 cigarette /3 month before pregnancy
	3=61-90
	4=91-180
	5=181-270
	6=171-450
	6=more than 450
Did the mother smoke	multiply the number of cigarette with the number of days in the three months

smoking tobacco in the 1st trimester	
	0=did not
	1=30 or less cigarette /3 month after pregnancy
	2=31-60
	3=61-90
	4=91-180
	5=181-270
	6=171-450
	6=more than 450
Did the mother smoke Sheesha 3 months before pregnancy	multiply the number of sheesha with the number of days in the three months
	0=did not
	1=<10 times smoked sheesha in 3 months before pregnancy
	2=11-30 times
	3=31-60 times
	more than 60
Did the mother smoke Sheesha in the 1st trimester	multiply the number of sheesha with the number of days in the three months
	0=did not
	1=<10 times smoked sheesha in 3 months after pregnancy
	2=11-30 times
	3=31-60 times
	more than 60
Did the father smoke smoking tobacco 3 months before pregnancy	multiply the number of cigarette with the number of days in the three months
	0=did not
	1=30 or less cigarette /3 month before pregnancy
	2=31-60

	3=61-90
	4=91-180
	5=181-270
	6=171-450
	6=more than 450
Did the father smoke smoking tobacco 3 months after pregnancy	multiply the number of cigarette with the number of days in the three months
	0=did not
	1=30 or less cigarette /3 month after pregnancy
	2=31-60
	3=61-90
	4=91-180
	5=181-270
	6=171-450
	6=more than 450
Did the father smoke Sheesha 3 months before pregnancy	multiply the number of sheesha with the number of days in the three months
	0=did not
	1=<10 times smoked sheesha in 3 months before pregnancy
	2=11-30 times
	3=31-60 times
	more than 60
Did the father smoke Sheesha in the 1st trimester	multiply the number of sheesha on the number of days in the three months
	0=did not
	1=<10 times
	2=11-30 times
	3=31-60 times
	more than 60

passive smoking	exposure to someone else's smoking during 3 months before or after conception
	1= yes
	2= no
Stress	
Family problems	including divorce, death in the family and conflicts during the 3 month before pregnancy and the 1st trimester
	1= yes
	2= no
Do you think you were under pressure during the 3 month before pregnancy and the 1st trimester?	
	1= yes
	2= no
sanguinity:	
are the parents relatives?	1= yes
	2= no
what is the relation between the parents	0=no relation
	1=1st degree cousin
	2=1st cousin once removed
	3=2nd degree cousin
	4=same tribe
Family History:	
Any other member of the family affected?	affected with any birth defect
	1= yes
	2= no
What is the defect this family member has	0=no

	1=CLP
	2=CP
	3=limb abnormality
	4=CV
	5=other orofacial defects
	6=genitourea
	7=multiple defects
	8=others
What is his relation to the child?	1=sibling
	2=parents
	3=grandparents
	4=uncle or aunt
	5=cousin
Examination	
Have photos been taken	1= yes
	2= no
Was there prenatal diagnosis	1= yes
	2= no
Does the child have Pierre Robin	It is a malformation or a sequence associated with micrognathia
	1= yes
Any associated abnormalities?	2= no
	1= yes
	2= no
What is the associated abnormality	0=no
	1=CV

	2=limbs malformation
	polydactyly
	Hydrocephaly
	UT
	other facial abnormality
	others
Type of cleft	
Is the cleft syndromic or non-syndromic	1=Syndromic
	2=nonsyndromic
Name of the syndrome	0=no syndrome
	1=Apert
	2=Achondroplasia
	3=Binder
	4=Cleft lip and Palate - Di George's
	5=Cleft lip and Palate - Pierre-Robin
	6=Cleido-cranal dysplasia
	7=Cranofacial microsomia
	8=Crouzon
	9=Goldenhar
	10=Hemifacial microsomia
	11=Mandibulofacial dysplasia (Treacher Collin's syndrome)
	12=Oro-facial-digital
Name of category of the cleft	
Simonart band	
Right side	1= yes
	2= no

left side	1= yes
	2= no
cleft lip	
Right side	1= yes
	2= no
left side	1= yes
	2= no
Alveolus	
Right side	1= yes
	2= no
left side	1= yes
	2= no
Hard Palate	1= yes
	2= no
Soft Palate	1= yes
	2= no

A10: Final list of included medical centers, principle investigator (PI), date of research approval request and acceptance.

City	Name of the Hospital	Person to contact	Date of request	Received Acceptance
Jeddah	King Fahad Hospital	Dr. Hussain Alamary	21/8/2010	20/10/2010
	Al-Mesaadia Maternity Hospital	Zamzam	21/8/2010	20/10/2010
	Al-Azeezia Maternity Hoapital	Ibtissam	21/8/2010	20/10/2010
	King Abdulaziz University Hospital	Prof. Najlaa Alamoudi	26/6/2010	26/7/2010
	King Abdulaziz Medical City (KAMC)	• Mamoon Daghistani • Danya • Dr. Mosleh	28/9/2010	12/12/2010
	King Fahad Military Hospital (KFMH)	Dr. Manal Almalik	1/12/2010	16/2/2011
Riyadh	King Fahad Medical City (KFMC)	Dr. Sari	7/9/2010	17/11/2010
	King Faisal Specialized Hospital (KFSH)	Dr. Al-Johar	21/8/2010	IRB rejected the request
	King Abdulaziz Medical City (KAMC)	Dr. Naser	27/12/2010	11/6/2011
	Maternity and Children Hospital+ King Saud Medical City (KSMC)	Dr. Mostafa	10/10/2010	25/3/2011
	King Fahad Armed Hospital	Dr. Eman	1/4/2011	10/8/2011
Maddina	Maternity and children Hospital	Dr. Fatma Daood	20/9/2010	2/2/2011

A11: DNA extraction using QAGEN Kit

Laboratory procedure to Extract DNA using Qagen kit:

- In a tube, add: Proteinase enzyme (20µl) + buffer (200µl) +saliva sample (200µl)
- Incubation 56^{o}C for 10 Min
- Centrifuge 20S
- Add to the mix: ethanol (200µl)
- Put on Vortex Maxi for 20 S to mix
- Put on centrifuge for 20 S
- Pour the mix in a *spin columns* tube that has Silica gel
- Centrifuge for 1 Min/8000RPM
- Add washing buffer to the mix twice. The first time with AW1 high concentration (500µl) then centrifuge 8000RPM for 1Min. the second time with AW2 low concentration (500µl) 14,000RPM for 2Min then 8000RPM for 4Min. In each time remove the leftover liquid in the tube
- Finally, add elution buffer and incubate for 30 Min.

A12: Research ethical approvals

Kingdom of Saudi Arabia
Ministry of Health
King Fahd Medical City

المملكة العربية السعودية
وزارة الصحة
مدينة الملك فهد الطبية

October 26th 2010
IRB#10-079

Dear Prof. Najla Alamoudi & Dr. Sari Rabah,

It is my pleasure to inform you that the IRB has recommended your submissions titled: **An investigation in the prevalence and etiology of Orofacial Clefts in Saudi Arabia** for approval.

Please be informed that in conducting this study, you as the Principal Investigator are required to abide by the rules and regulations of the Government of Saudi Arabia and KFMC/IRB. Further, you are required to submit a Progress Report before September 26th 2011 it can be reviewed by the IRB without lapse of approval. The approval of this proposal will automatically be suspended on October 26th 2011 pending the acceptance of the Progress Report. You also need to notify the IRB as soon as possible in the case of:

1. Any amendments to the project;
2. Termination of the study;
3. Any serious unexpected adverse events (within two working days).
4. Any event or new information that may affect the benefit/risk ratio of the proposal.

Please observe the following:

1. Personal identifying data should only be collected when necessary for research;
2. The data collected should only be used for this proposal;
3. Data should be stored securely so that a few authorized users are permitted access to the database;
4. Secondary disclosure of personal identifiable data is not allowed.
5. Copy of the Consent Form should be kept in the Research Subject's Medical Record and the consent process should be documented in the medical record.

We wish you every success in your research endeavour.

If you have any further questions feel free to contact me.

F M
Approved
26 OCT 2010
H 10-079
I R B

Sincerely yours,

Prof. Faroque Ahmad Khan, MB, MACP

Chairman Institutional Review Board–IRB.
King Fahd Medical City, Riyadh, KSA.
Tel: + 966 1 288 9999 Ext. 7185 / 1299
E-mail: fakhan@kfmc.med.sa
faroquekhan@yahoo.com

الرقم : _____ المرفقات : _____

التاريخ : _____

KING FAHD ARMED FORCES HOSPITAL
P.O. BOX : 9862 JEDDAH 21159
KINGDOM OF SAUDI ARABIA

مستشفى الملك فهد للقوات المسلحة
ص . ب : ٩٨٦٢ جـــــدة ٢١١٥٩
المملكـة العـربيـة السعـوديـة

8 February 2011

Dr Heba Sabbagh
Specialist and Lecturer
King Abdulaziz University
Jeddah

Dear Dr Sabbagh,

RE: Your research proposal re The Prevalence and Etiology of Cleft Lip and Palate

Thank you for considering the hospital as one of your bases in your study.

Your research proposal was discussed in our committee meeting last 31st January 2011. The committee has no objection in approving your research proposal provided that the copy of parental consent is presented to the committee and time frame of the study will be amended to the current year

Once the above are completed, the committee will allow the commencement of your study. Dr Manal Malek, Consultant Pedodontcs ~~Consultant~~ will be your coordinator at the hospital for this study. She can be reached through her number in the Dental Clinic, i.e. 665 3000ext 3496.

We hope that you would be able to submit the above requirements.

Sincerely,

Dr K F Al Shaibi
Consultant Cardiologist
Deputy Director of Cardiac Services
Chairman of Research and Ethics Committee

/kas/lp/0211/031

Copy: Lt Col (Dr) Y Rehbini, Director of Dental Services/Actg Director of
Academic Affairs and Training

M.S.D. Printing Press 001-37925

KINGDOM OF SAUDI ARABIA
Ministry of Higher Education
KING ABDULAZIZ UNIVERSITY
Faculty of Medicine

المملكة العربية السعودية
وزارة التعليم العالي
جامعة الملك عبد العزيز
كلية الطب

Ref FM :
Date : / /
Encl :

الرقم : ٥/٣١/٥٨٧٦
التاريخ : ٥ / ٦ / ١٤٣١هـ
المرفقات :

UNIT OF
BIOMEDICAL ETHICS
Research Committee

TO : Dr. Ail Habiballah Hassan
From : Professor H Nasrat
Date :WEDNESDAY, MAY 12, 2010
CC : Vice-Dean, University /Hospital Director& File
RE : Prevalence and genetic characteristic of deft lip and palate Patients .(Reference No 359-10)

THIS IS TO CERTIFY THAT RESEARCH TITLED :
Prevalence and genetic characteristic of deft lip and palate Patients .
Submitted by :
Dr. Ail Habiballah Hassan **Faculty of Dentistry (Orthodontics Dept.)**

Has been reviewed by the committee with respect to protecting the rights welfare of human subjects involved in the research project and / or experimental animals utilized. The methods employed are adequate for obtaining the information required and satisfy the required ethical principles and does not involve undue risk in the light of the medical benefits to derived there form .

Decision :
The committee approves the above mentioned proposal as fulfilling the ethical requirements .

Professor Hassan A Nassrat

Chairman of the Bioethical

Mohammed al searee (Reference No 359-10)

ص.ب ٨٠٢٠٥ جدة ٢١٥٨٩ برقياً : «جامعة عبدالعزيز» تلكس ٦٠١١٤١ كايوني إس جيه فاكس : ٦٤٠٠٨٥٥ ٦٩٥٢٤٤٦/٦٩٥٢٠٦٣
P.o. Box 80205 Jeddah 21589 Cable : "Jameatabdulaziz" Telex : 601141 Kauni SJ Fax. : 6400855 ☎ : 6952446/6952063

RIYADH MILITARY HOSPITAL
P.O. Box 7897, Riyadh 11159
Kingdom of Saudi Arabia

Research & Ethics Committee

01 August 2011

DR. EMAN AL NAMANKANI
Orthodontist
Department of Dentistry

Re: An investigation in the prevalence and etiology of orofacial clefts in Saudi Arabia

Dear Dr. Al Namankani

The members of Research and Ethics Committee have reviewed the abovementioned research proposal. On behalf of the committee, I am pleased to inform you that this research project has now been approved as chairman action and documented under:

Your research protocol has now been documented under:

Project No.	429
Series of	2011

Kindly quote the project number indicated herein in all transactions and communications. You are advised *to submit a 6-monthly report* in relation to this research project to update the committee of its progress.

I trust your research project proves fruitful and beneficial to RMH.

Yours sincerely

DR. SAEED KADASAH
Chairman, Research and Ethics Committee
First Floor, Building 136

Ministry of Health
Directorate of Health Affairs – Jeddah

H./E. The Supervisor of King Abdulaziz Hospital & Oncology Center Program

Greetings,

Kindly be informed that researcher Dr./ Heba Sabbagh will conduct a research titled:

"Incidence and Etiology of Cleft Lip and Palate in Saudi Arabia"

Having reviewed and considered the methodology of the research by the Scientific Committee and the National Committee of Biological & Medical Ethics, vide No. (H-02-J-002), it was found that there is no objection to conduct the said research.

I kindly request to facilitate the mission of the researcher and assist her to conduct the research, taking into consideration that the service in the concerned utilities will not be affected, the rights and privacy of persons under the research are strictly observed and that the information are only used for the purposes of the scientific research, knowing that the approval is valid for two years from the date thereof.

Your cooperation is much appreciated.

Best regards,

Assistant Director of Health Affairs for Planning & Development
Jeddah Province
Dr./ Osama Obaid Thafar

No.: C/47/302/38340 **Date: 23/11/1431H.** **Enclosures: Non**

Kingdom of Saudi Arabia
National Guard
Health Affairs
King Abdulaziz Medical City - Jeddah

المملكة العربية السعودية
رئاسة الحرس الوطني
الشئون الصحية
مدينة الملك عبدالعزيز الطبية - جدة

Research Committee, KAMC, WR
☎ : (02) 624-0000 x 21891 / ✉ : 6466

INTERNAL MEMORANDUM

To	:	Prof. Najla Al Amoudi Head of Preventive Dental Services Dept. King Abdulaziz University, Jeddah
Date	:	12th December 2010G / 06th Muharram.1432H
Study title	:	**An investigation in the prevalence and etiology of orofacial Clefts in Saudi Arabia**
HRERC ref. #	:	**RCJ1010-153**

Please be advised that your research proposal entitled above submitted in this office on the 18th of October 2010 has received favorable opinion from the committee and thereby giving you approval to conduct your study.

Condition of approval:-

- This notice of acceptance is based on the approved application, protocol and supporting documentation. Any significant deviations or unanticipated developments within the research study should be brought to the attention of the Hospital Research Committee for subsequent approval.
- This approval is valid for "**1 year**" in which you have to apply for re-approval should you need further extension.

Kindly note that we may, for the purpose of audit, contact you from time to time to ascertain the status of your study and we do hope in due course to be informed of the progress and final outcome of the study once it is completed.

Best wishes for the successful completion of your study.

Kind regards,

Dr. Abdulfattah Al-Amri
A/Chairman, Research Committee
Clinical Scientist, Path & Lab Med. Dept.
King Abdulaziz Medical City, W.R.

cc: Dr. M. Dughestani, Coor., KAMC, Jeddah
Dr. H. Sabbagh, Specialist, KAU, Jeddah

c:\Amedoc\kamc\studies\RCJ1010-153\approval.doc

P.O.BOX 9515
JEDDAH 21423
KINGDOM OF SAUDI ARABIA
FAX : 624 7444
TEL.: 624 0000
فاكس : ٦٢٤٧٤٤٤
تليفون : ٦٢٤٠٠٠٠
ص.ب ٩٥١٥
جدة ٢١٤٢٣
المملكة العربية السعودية

Kingdom of Saudi Arabia
National Guard Health Affairs

المملكة العربية السعودية
الحرس الوطني - الشؤون الصحية

Research Office 14572/14528 1515 2520772 researchoffice@ngha.med.sa

MEMORANDUM

Ref. #: RO/296/2012

Date: **(G) 29 APRIL 2012**
(H) 08 Jumada Al-Akhir 1433

To: **DR. NASIR ALHAMLAN**
Principal Investigator
Consultant and Program Supervisor
Orthodontic Division
National Guard Health Affairs

From: **DR. MAJED AL JERAISY**
Chairman, Research Committee
King Abdullah International Medical Research Center
National Guard Health Affairs

Majed 2/5/12

Subject: **Protocol RCJ1010-153** -"An Investigation in the Prevalence and Etiology of Orofacial Clefts in Saudi Arabia"

Thank you for submitting the above-mentioned subject, which has been studied by the Research Committee Chairman, after careful review we have decided to **award scientific approval for your PhD project**.

Your proposal will be forwarded to the Institutional Review Board (IRB) for extension of IRB approval and to add KAMC- Central region to your study area.

You should not start your project until this approval from IRB has been granted.

Please do not hesitate to call our office at Ext. 14572/14528, if you have any questions.

Thank you.

A13: Distribution of the sample according to cleft phenotype and hospital of referral.

City	Hospital	CL (%)	CLP (%)	CP (%)	NSOFC (%)	Control
Jeddah 71 cases 104 controls	King Fahad hospital and Mossadia Maternity Hoswpital	5 (29.4)	5 (29.4)	7 (41.2)	17 (100)	24
	King Abdulaziz University Hospital	5 (25)	11 (55)	4 (20)	20 (100)	30
	King Abdulaziz Medical City	7 (31.8)	14 (63.6)	1 (4.5)	22 (100)	33
	King Fahad Armed Hospital	7 (77.8)	1 (11.1)	1 (11.1)	9 (100)	12
	Alazizia Maternity Hospital	2 (66.7)	0	1 (33.3)	3 (100)	5
Riyadh 100 case 103 controls	King Fahad Medical City	5 (29.4)	5 (29.4)	7 (41.2)	17 (100)	17
	King Saud Medical City	14 (46.7)	8 (26.7)	8 (26.7)	30 (100)	33
	Riyadh Armed Hospital	11 (44)	11 (44)	3 (12)	25 (100)	24
	Riyadh NGH	7 (25)	10 (35.7)	11 (39.3)	28 (100)	29
Maddina 34 cases 37 controls	Maddina maternity and children hospital	15 (44.1)	9 (26.5)	10 (29.4)	34 (100)	37
Total		78 (38)	74 (36.8)	53 (25.8)	205* (100)	244

*The number is less than the total number of cases (208) as the phenotype for three cases was missing.

A14: Distribution of the sample according to child's nationality

Countries	Study (%)	Control (%)	Total (%)
Saudi	183 (88.4)	214 (87.7)	400 (88.1)
Other Middle East countries	16 (7.7)	15 (6.1)	31 (6.8)
Asian country	6 (2.9)	6 (2.5)	12 (2.6)
North Africa	2 (1)	5 (2)	7 (1.5)
Other African countries	0	4 (1.6)	4 (0.9)
Total	208 (100)	244 (100)	454 (100)

P= 0.32

A15: Case-control comparison according to cleft phenotype and maternal ingestion of multivitamins in the pre-gestation and 1st trimester periods.

Duration	Multi-vitamins use	NSOFC (%)	CL (%)	CLP (%)	CP (%)	Controls (%)
Pre-gestation	Yes	9 (4.4)	3 (3.9)	5 (6.8)	1 (1.9)	8 (3.3)
X^2 =2.37	No	194 (95.6)	73 (96.1)	69 (93.2)	52 (98.1)	236 (96.7)
df=3	Total	203[C] (100)	76[C] (100)	74 (100)	53 (100)	244 (100)
P= 0.5	**P-value**	**0.401**	**0.79**	**0.19**	**0.58**	
	OR (95% CI)	1.5 (0.58–3.9)	1.2 (0.31–4.67)	2.12 (0.67–6.69)	0.56 (0.07–4.58)	
1st trimester	Yes	38 (18.8)	13 (17.1)	12 (16.4)	13 (24.5)	53 (21.7)
X^2 =2.4	No	164 (81.2)	63 (82.9)	61 (83.6)	40 (75.5)	191 (78.3)
df=3	Total	202[C] (100)	76[C] (100)	73[C] (100)	53 (100)	244 (100)
P= 0.494	**P-value**	**0.465**	**0.38**	**0.31**	**0.656**	
	(95% CI)	0.84 (0.53–1.3)	0.74 (0.38–1.45)	0.7 (0.35–1.4)	1.15 (0.58–2.31)	

[C] number less than the total sample (NSOFC: 205; CL; 78 and CLP: 74) because of missing information

A16: Case-control comparison according to cleft sub-phenotype and maternal ingestion of iron during the three month pre-gestation and 1st trimester periods.

Duration	Iron use	NSOFC (%)	CL (%)	CLP (%)	CP (%)	Controls (%)
Pre-gestation	Yes	15 (7.4)	7 (9.2)	6 (8.2)	2 (3.8)	12 (4.9)
X^2 =1.5	No	187 (92.6)	69 (90.8)	67 (91.8)	51 (96.2)	232 (95.1)
df=3	Total	202[C] (100)	76[C] (100)	73[C] (100)	53 (100)	244 (100)
P= 0.683	**P-value**	**0.213**	**0.17**	**0.29**	**0.71**	
	OR (95% CI)	1.63 (0.75–3.53)	1.95 (0.74–5.15)	1.72 (0.62–4.75)	0.75 (0.16–3.45)	
1st Trimester	Yes	69 (34.1)	25 (32.9)	24 (32.9)	20 (37.7)	92 (37.7)
X^2 =5.04	No	133 (65.9)	51 (67.1)	49 (67.1)	33 (62.3)	152(62.3)
df=3	Total	202 (100)	76 [C] (100)	73 [C] (100)	53 (100)	244 (100)
P= 0.169	**P-value**	**0.353**	**0.43**	**0.43**	**0.95**	
	OR (95% CI)	0.83 (0.56–1.23)	0.81 (0.47–1.39)	0.8 (0.46–1.39)	0.98 (0.53–1.8)	

[C] number less than the total sample (NSOFC: 205; CL; 78 and CLP: 74) because of missing information

A17: Case-control comparison according to cleft phenotype and maternal renal disease during the three month pre-gestation and the1st trimester periods.

Duration	Renal disease	NSOFC (%)	CL (%)	CLP (%)	CP (%)	Controls (%)
Pre-gestation	Yes	6 (3.4)	1 (1.3)	2 (2.7)	3 (5.7)	4 (1.7)
X^2 =3.54	No	198 (96.6)	76 (98.9)	72 (97.7)	50 (94.3)	238 (98.3)
df= 3	Total	204[C] (100)	77[C] (100)	74 (100)	53 (100)	242 (100)
P= 0.316	**P-value**	**0.234**	**0.84**	**0.56**	**0.09**	
	OR (95% CI)	2.09 (0.6–7.25)	0.8 (0.09–7.28)	1.67 (0.3-9.34)	3.53 (0.77–16.24)	
1st trimester	Yes	6 (2.9)	4 (5.3)	0	2 (3.8)	8 (3.3)
X^2 =3.55	No	198 (97.1)	73 (94.7)	74 (100)	51 (96.2)	234 (96.7)
df= 3	Total	204[c] (100)	77[c] (100)	74 (100)	53 (100)	242 (100)
P= 0.314	**P-value**	**0.957**	**0.452**	**0.252**	**0.88**	
	OR (95% CI)	1.03 (0.37–2.9)	1.6 (0.48–5.61)	a	1.13 (0.23–5.49)	

a. The OR and CI were not measured because one group contains zero value.

[C] Number less than the total cases (NSOFC: 205; CL: 78; and CP:53) because of missing information.

A18: Case-control comparison according to cleft phenotype and maternal asthma during the three month pre-gestation and 1st trimester periods.

Duration	Asthma	NSOFC (%)	CL (%)	CLP (%)	CP (%)	Controls (%)
Pre-gestation X^2 =2.63 df= 3 P= 0.452	Yes	5 (2.4)	1 (1.3)	3 (4.1)	1 (1.9)	3 (1.2)
	No	199 (97.6)	76 (98.7)	71 (95.9)	52 (98.1)	239 (98.8)
	Total	204 [c] (100)	77[c] (100)	74 (100)	53 (100)	242 (100)
	P-value	**0.34**	**0.968**	**0.143**	**0.714**	
	OR (95% CI)	1.9 (0.47–8.4)	1.05 (0.11–10.46)	3.39 (0.67–17.15)	1.5 (0.15–14.84)	
1st trimester X^2 =0.49 df= 3 P= 0.92	Yes	4 (1.9)	1 (1.3)	2 (2.7)	1 (1.9)	6 (2.5)
	No	202 (98.1)	76 (98.7)	72 (97.3)	52 (98.1)	233 (97.5)
	Total	206 (100)	77 (100)	74 (100)	53 (100)	239 (100)
	P-value	**0.701**	**0.55**	**0.91**	**0.79**	
	OR (95% CI)	0.78 (0.22–2.8)	0.53 (0.06–4.47)	1.1 (0.22–5.56)	0.75 (0.09–6.34)	

[C] Number less than the total cases (NSOFC: 205; CL: 78; and CP:53) because of missing information.

A19: Case-control comparison according to maternal convulsions during the 3 month pregestation p**eriod.**

Convulsions	**NSOFC (%)**	**CL (%)**	**CLP (%)**	**CP (%)**	**Controls (%)**
Yes	1 (0.4)	0	1 (1.4)	0	1 (0.4)
No	204 (99.6)	78 (100)	73 (98.6)	53 (100)	238 (99.6)
Total	205 (100)	78 (100)	74 (100)	53 (100)	239[C] (100)
P-value	**0.91**	**0.57**	**0.37**	**0.64**	
OR (95% CI)	1.17 (0.073–18.8)	a	3.32 (0.21–53.7)	a	

X^2 =6.65, df= 3, P= 0.585

a. The OR and CI were not measured because one group contains zero value.
[C] Number less than the total number (controls: 244) because of missing information.

A20: Case-control comparison according to maternal high blood pressure during the three months pre-gestation and 1st trimester periods.

Duration	High blood pressure	NSOFC (%)	CL (%)	CLP (%)	CP (%)	Controls(%)
Pre-gestation X^2 =0.84 df= 3 P= 0.84	Yes	4 (1.9)	1 (1.3)	2 (2.7)	1 (1.9)	4 (1.7)
	No	201 (98.1)	77 (98.7)	72 (97.3)	52 (98.1)	238 (98.3)
	Total	205 (100)	78 (100)	74 (100)	53 (100)	242[C] (100)
	P-value	**0.824**	**0.819**	**0.573**	**0.91**	
	OR (95% CI)	1.17 (0.29–4.75)	0.77 (0.09–7.02)	21.65 (0.3–9.2)	1.14 (0.13–10.45)	
1st trimester X^2 =1.91 df= 3 P= 0.592	Yes	5 (2.4)	1 (1.3)	3 (4.1)	1 (1.9)	5 (1.7)
	No	200 (97.6)	77 (98.7)	71 (95.9)	52 (98.1)	237(98.3)
	Total	205(100)	78 (100)	74 (100)	53 (100)	242[C] (100)
	P-value	**0.93**	**0.66**	**0.226**	**0.914**	
	OR (95% CI)	0.94 (0.25–3.54)	0.62 (0.07–5.35)	2.48 (0.54–11.35)	1.14 (0.12–10.3)	

[C] Number less than the total number (controls: 244) because of missing information.

A21: Case-control comparison according to cleft phenotype and vaginal bleeding during the 1st trimester.

vaginal bleeding	NSOFC (%)	CL (%)	CLP (%)	CP (%)	Controls (%)
Yes	15 (7.4)	4 (5.3)	7 (9.6)	4 (7.5)	10 (4.1)
No	189 (92.6)	73 (94.7)	67 (90.4)	49 (92.5)	232 (95.9)
Total	204[C] (100)	77 [C] (100)	74 (100)	53 (100)	242[C] (100)
P-value	**0.1**	**0.692**	**0.07**	**0.3**	
OR (95% CI)	2 (0.87–4.4)	1.3 (0.4–4.28)	2.44 (0.89–6.66)	1.87 (0.56–6.21)	

X^2 =3.37, df=3 and P= 0.338

[C] Number less than the total number (NSOFC: 205 cases, CL: 78 cases and controls: 244) because of missing information.

A22: Case-control comparison according to cleft phenotype and maternal use of contraceptives in the pre-gestation and 1st trimester periods.

Duration	Contra-ceptives	NSOFC (%)	CL (%)	CLP (%)	CP (%)	Controls (%)
Pre-gestation	Yes	15 (7.8)	3 (3.9)	7 (9.7)	5 (9.4)	27 (11.1)
X^2 =3.73	No	189 (92.2)	75 (96.1)	66 (90.3)	48 (90.6)	217 (88.9)
df=3	Total	204[C] (100)	78 (100)	73[C] (100)	53 (100)	244 (100)
P= 0.292	**P-value**	**0.249**	**0.069**	**0.721**	**0.71**	
	OR (95% CI)	0.68 (0.36–1.31)	0.32 (0.1–1.09)	0.85 (0.36–2.05)	0.83 (0.3–2.25)	
1st trimester	Yes	3 (1.5)	1 (1.3)	1 (1.4)	1 (1.9)	6 (2.5)
X^2 =1.5	No	202 (98.5)	77 (98.7)	73 (98.9)	52 (98.1)	238 (97.5)
df=3	Total	205 (100)	78 (100)	74 (100)	53 (100)	244 (100)
P= 0.884	**P-value**	**0.449**	**0.55**	**0.57**	**0.79**	
	OR (95% CI)	0.59 (0.15–2.37)	0.53 (0.06–4.45)	0.55 (0.07–4.61)	0.75 (0.09–6.39)	

[C] number less than the total sample (NSOFC: 205; and CLP: 74) because of missing information

A23: Case-control comparison according to cleft phenotype and maternal use of progesterone tablet in the pre-gestation and 1st trimester periods.

Progesterone	**NSOFC (%)**	**CL (%)**	**CLP (%)**	**CP (%)**	**Controls (%)**
Yes	13 (6.3)	5 (6.4)	6 (8)	2 (3.8)	24 (10)
No	192 (93.7)	73 (93.6)	68 (92.0)	51 (96.2)	217 (90)
Total	205 (100)	78 (100)	74 (100)	53 (100)	241[C] (100)
P value	**0.171**	**0.347**	**0.636**	**0.168**	
OR (95% CI)	0.61 (0.3-1.24)	0.62 (0.23-1.68)	0.8 (0.31-2.03)	0.35 (0.08-1.55)	

X^2=2.55, df=2 and P= 0.279

[C] number less than the total controls (244) because of missing information

A24: Case-control comparison according to cleft phenotype and maternal experience of threatened abortion in the 1st trimester period.

Threatened abortion	NSOFC (%)	CL (%)	CLP (%)	CP (%)	Controls (%)
Yes	12 (6.3)	4 (5.3)	6 (8.2)	2 (3.8)	11 (4.5)
No	192 (93.7)	73 (94.7)	68 (91.8)	51 (96.2)	231 (95.5)
Total	204[C] (100)	77[C] (100)	74 (100)	53 (100)	242 (100)
P-value	**0.533**	**0.815**	**0.241**	**0.79**	
OR (95% CI)	1.31 (0.56–3.03)	1.18 (0.36–3.81)	1.86 (0.67–5.23)	0.804 (0.18–3.78)	

X^2 =1.66, df=3, P= 0.654

A25: Case-control comparison according to cleft phenotype and severe morning sickness in the 1st trimester period.

Severe morning sickness	NSOFC (%)	CL (%)	CLP (%)	CP (%)	Controls (%)
Yes	21 (10.3)	8 (10.7)	6 (8.2)	7 (13.2)	20 (8.3)
No	183 (89.7)	69 (89.3)	68 (91.8)	46 (868)	222 (91.7)
Total	204[C] (100)	77[C] (100)	74 (100)	53 (100)	242 (100)
P-value	**0.38**	**0.567**	**0.966**	**0.27**	
OR (95% CI)	1.33 (0.7–2.5)	1.29 (0.54–3.05)	0.98 (0.38–2.55)	1.67 (0.67–4.17)	

X^2 =1.44, df= 3, P= 0.7

[C] number less than the total sample (NSOFC: 205; and CL:78) because of missing information

A26: Case-control comparison according to NSOFC phenotype and maternal abdominal pain in the three month pre-gestation and 1st trimester periods.

Duration	Pain	NSOFC (%)	CL (%)	CLP (%)	CP (%)	Controls (%)
Pre-gestation X^2 =3.05 df=3 P= 0.385	Yes	10 (4.9)	3 (4)	4 (5.7)	3 (5.7)	5 (2.1)
	No	194 (95.1)	74 (96)	70 (94.3)	50 (94.3)	237 (97.9)
	Total	204[C] (100)	77[C] (100)	74 (100)	53 (100)	242 (100)
	P-value	**0.100**	**0.379**	**0.126**	**0.15**	
	OR (95% CI)	2.43 (0.82–7.23)	1.97 (0.46–8.4)	2.71 (0.7–10.36)	2.81 (0.65–12.13)	
1st trimester X^2 =8.15 df=3 P= 0.043**	Yes	23 (11.2)	7 (8)	7 (9.6)	9 (17)	14 (5.8)
	No	181 (88.8)	70 (92)	67 (90.4)	44 (83)	228 (94.2)
	Total	204[C] (100)	77[C] (100)	74 (100)	53 (100)	242 (100)
	P-value	**0.041****	**0.312**	**0.272**	**0.004****	
	OR (95% CI)	2.06 (1.03–4.11)	1.63 (0.63–4.19)	1.7 (0.7–4.39)	3.56 (1.43–8.83)	

** Significance value P≤ 0.05

[C] number less than the total sample (NSOFC: 205; and CL: 78) because of missing information

A27: Distribution of the sample according to cleft phenotypes and; their relationship to paternal waterpipe smoking types (Moasel and Jorak smoking); and maternal second hand paternal smoking, in the three month pregestation and 1st trimester period

A27.1 case-controls comparison according to cleft phenotype and paternal Moasel smoking in the three month pregestation and 1st trimester period

Period	Paternal Moasel	NSOFC (%)	CL (%)	CLP (%)	CP (%)	Controls (%)
Three month pregestation	Yes	15 (7.3)	6 (8)	8 (10.8)	1 (1.9)	12 (4.9)
	No	190 (92.7)	70 (92)	66 (89.2)	52 (98.1)	232 (95.1)
	Total	203[C] (100)	76[C] (100)	74 (100)	53 (100)	244 (100)
	P value	0.287	0.338	0.072	0.321	
	OR (CI)	1.53 (0.7-3.34)	1.64 (0.59-4.52)	2.31 (0.91-6)	0.37 (0.05 2.89)	
1st trimester	Yes	10 (5)	7 (5.3)	8 (10.8)	0	9 (3.7)
	No	191 (95)	69 (94.7)	66 (89.2)	53 (100)	234 (96.3)
	Total	203[C] (100)	76[C] (100)	74 (100)	53 (100)	243 (100)
	P value	0.51	0.178	0.087	0.15	
	OR (CI)	1.36 (0.54-3.42)	1.93 (0.73-5.09)	2.22 (0.87-5.66)	1.23 (1.16 1.3)	

[C] Number less than the total number (NSOFC: 205 cases, and CL: 78 cases) because of missing information.

A27.2 case-controls comparison according to cleft phenotype and paternal Jorak smoking in the three month pregestation and 1st trimester period

Period	Smoking	NSOFC (%)	CL (%)	CLP (%)	CP (%)	Controls (%)
pregestation	Yes	15 (7.3)	5 (6.7)	8 (10.8)	1 (1.9)	3 (1.2)
	No	190 (93)	71 (93.3)	66 (89.2)	52 (98.1)	241 (98.8)
	Total	203[C] (100)	76[C] (100)	74 (100)	53 (100)	244 (100)
	P value	0.001**	0.01**	0.000**	0.715	
	OR (CI)	6.34 (1.8-22.23)	5.58(1.3-23.95)	9.62 (2.48-37.27)	1.53 (0.16-14.96)	
1st trimester	Yes	11 (5.5)	4 (5.4)	6 (8.2)	1(1.9)	3 (1.2)
	No	189 (94.5)	70 (94.6)	67 (91.8)	52 (98.1)	240 (98.8)
	Total	200[C] (100)	74[C] (100)	73[C] (100)	53 (100)	243 (100)
	P value	0.01**	0.03**	0.002**	0.72	
	OR (CI)	4.66 (1.28-16.93)	4.55 (1-2-.82)	7.1 (1.73-29.16)	1.52 (0.16-14.9)	

** Significant value P<0.05

[C] Number less than the total number (NSOFC: 205 cases, and CL: 78 cases) because of missing information.

A27.3 Case-control comparison according to cleft phenotype and maternal second-hand smoking among fathers using smoking tobacco in the pregestation and/or 1st trimester periods

	Maternal 2nd hand smoking	**CL (%)**	**CLP (%)**	**CP (%)**	**Controls (%)**
Fathers using smoking tobacco cases=57 controls=**77**	Yes	7 (77.8)	18 (72)	13 (56.5)	35 (46.1)
	No	2 (22.2)	7 (28)	10 (43.5)	42 (53.9)
	Total	9 (100)	25 (100)	23 (100)	77 (100)
	OR (95% CI)	4.1 (0.8-21.03)	10.54** (2.29-48.64)	15.23** (1.9-122.3)	

X^2 =7.28, df=3 and P= 0.063

A28: Case-control comparison according to cleft phenotype and maternal exposure to pesticides during the three month pre-gestation and 1st trimester periods.

Duration	Pesticides	NSOFC (%)	CL (%)	CLP	CP (%)	Controls (%)
Pre-gestation X^2=1.52 df= 3 P= 0.678	Yes	31(15.1)	9 (11.8)	13 (17.6)	9 (17)	42 (17.2)
	No	173 (84.9)	68 (88.2)	61 (82.4)	44 (83)	202 (82.8)
	Total	204[C] (100)	77[C] (100)	74 (100)	53(100)	244 (100)
	P-value	**0.55**	**0.248**	**0.978**	**0.938**	
	OR (95% CI)	0.86 (0.52 - 1.42)	0.64 (0.29 - 1.38)	1.01 (0.51 - 2)	0.97 (0.44 - 2.14)	
1st trimester X^2=0.66df = 3 P= 0.882	Yes	37 (18.1)	12 (15.8)	14 (18.9)	11 (20.8)	43 (17.6)
	No	167 (81.9)	65 (84.2)	60 (81.1)	42 (79.2)	201 (82.4)
	Total	204[C] (100)	77[C] (100)	74 (100)	53 (100)	244 (100)
	P-value	**0.99**	**0.741**	**0.769**	**0.568**	
	OR (95% CI)	1 (0.61 - 1.6)	0.89 (0.44 - 1.79)	1.11 (0.57 - 2.16)	1.24 (0.59 - 2.61)	

[C] number less than the total sample (NSOFC: 205; and CL: 78) because of missing information

A29: Distribution of the sample according to maternal exposure to microwaves and computers in the three month pregestation and the 1st trimester

A29.1: Distribution of the sample according to maternal exposure to microwaves in the three month pregestation and the 1st trimester

Period	Microwaves use	NSOFC (%)	CL (%)	CLP (%)	CP (%)	Controls (%)
Three month pregestation	Yes	84 (40.8)	34 (44.7)	25 (33.8)	22 (41.5)	117 (48)
	No	122 (59.2)	42 (55.3)	49 (66.2)	31 (58.5)	127 (52)
	Total	206 (100)	76 (100)	74 (100)	53 (100)	244 (100)
	P value	0.13	0.605	0.03**	0.38	
	OR (95% CI)	0.75 (0.5-1.1)	0.87 (0.52-1.46)	0.55 (0.32-0.95)	0.77 (0.42-1.4)	
1st trimester	Yes	84 (40.8)	36 (47.4)	24 (32.4)	23 (43.4)	117 (48)
	No	122 (59.2)	40 (52.6)	50 (67.6)	30 (56.6)	127 (52)
	Total	203[C] (100)	76[C] (100)	74 (100)	53 (100)	244 (100)
	P value	0.127	0.958	0.021**	0.57	
	OR (CI)	0.75 (0.5-1.1)	0.99 (0.59-1.65)	0.52 (0.30-0.91)	0.84 (0.46-1.5)	

** Significant value P<0.05

[C] Number less than the total number (NSOFC: 205 cases, and CL: 78 cases) because of missing information.

A29.2: Distribution of the sample according to maternal use of Computer in the three month pregestation and 1st trimester

Period	Computer use	NSOFC (%)	CL (%)	CLP (%)	CP (%)	Controls (%)
Three month pregestation	Yes	54 (26.3)	21 (27.6)	18 (24.7)	14 (26.4)	133 (29.6)
	No	151 (73.7)	55 (72.4)	55 (75.3)	39 (73.6)	316 (70.4)
	Total	202[C] (100)	76[C] (100)	73 (100)	53 (100)	449 (100)
	P value	0.16	0.478	0.235	0.43	
	OR (CI)	0.75 (0.5-1.13)	0.81 (0.46-1.44)	0.7 (0.38-1.27)	0.77 (0.39-1.49)	
1st trimester	Yes	50 (24.3)	19 (25)	17 (23)	13 (24.5)	75 (30.7)
	No	155 (75.)7	57 (75)	57 (77)	40 (75.5)	169 (69.3)
	Total	203[C] (100)	76[C] (100)	74 (100)	53 (100)	244 (100)
	P value	0.13	0.376	0.223	0.4	
	OR (CI)	0.72 (0.48-1.1)	0.77 (0.43-1.38)	0.69 (0.37-1.26)	7(0.38-1.48)	

[C] Number less than the total number (NSOFC: 205 cases, and CL: 78 cases) because of missing information.

A30: P values for variables that showed statistical significant relationship with NSOFC, CL, CLP and/or CP in chi square, but were not statistically significant in logistic regression analysis.

Variables	NSOFC	CL	CLP	CP
Singletons Vs. Twins	0.39	0.912	0.12	a
Antibiotic pregestation	0.063	a	0.14	a
Anti-emetic medication	a	0.131	a	a
Folic acid 1st trimester	0.103	a	a	a
Multivitamins 1st trimester	0.901	a	a	a
Illness pregestation	0.14	0.44	0.45	0.287
Illness 1st trimester	0.18	a	a	a
Common cold/flu pregestation	a	0.295	a	0.376
Common cold/flu 1st trimester	0.64	a	a	a
Fever pregestation	.0.4	a	0.33	a
Mother under stress	a	0.912	a	a
Family problems	0.37	a	0.85	0.604
Abdominal pain 1st trimester	0.21	a	a	a
Paternal water pipe smoking	a	0.23	a	a
Maternal incense exposure pregestation	a	0.502	a	a
Chemical 1st trimester	a	a	0.12	a
Solvent pregestation	a		0.33	a

a. No values either because the variable were entered in the logistic regression or had significant relationship with cleft.

A31: Distribution of infant *VAX1* rs4752028 genotypes in cases and control according to parental consanguinity.

Consanguinity	TT*	CC	CT	Total
Yes (%)	133 (57.8)	10 (76.9)	35 (45.5)	178 (55.6)
No (%)	97(42.2)	3(23.1)	42 (54.4)	142 (44.4)
Total (%)	230 (100)	13 (100)	77(100)	320(100)

X^2=6, df=2, P= 0.043**

* Homozygous common allele genotype

**Significant at the 0.05 level

A32: Distribution of infant *VAX1* rs4752028 genotypes in cases and controls according to parental consanguinity.

Consanguinity	**NSOFC**			**CL/P**			**CP**			**Control**		
	TT*	CC	CT	TT*	CC	CT	TT*	CC	CT	TT*	CC	CT
Yes (%)	56 (60.9)	7 (70)	24 (45.1)	42 (60)	6 (66.7)	17 (43.6)	14 (63.6)	1 (100)	6 (50)	77 (55.8)	3 (100)	12 (46.2)
No (%)	36 (39.1)	3 (30)	28 (54.9)	28 (40)	3 (33.3)	22 (56.4)	8 (36.4)	0	6 (50)	61 (54.2)	0	14 (55.8)
Total (%)	92 (100)	10 (100)	51 (100)	70 (100)	9 (100)	39 (100)	22 (100)	1 (100)	12 (100)	138 (100)	3 (100)	26 (100)
Total infants	153[C]			118[C]			35[C]			167[C]		
***X* (df), P-value**	**4.19 (2), 0.123**			**4.88 (2), 0.087**			**1.89 (2), 0.39**			**3.31 (2), 0.19**		

* Homozygous common allele genotype

[C] Out of 120 CL/P, 35 CP, and 188controls recorded, two CL/P, and 22 controls did not have their genotyping and/or paternal consanguinity information completed

A33: Comparison between case and control infant *VAX1* rs4752028 genotypes and their relationship to parental consanguinity.

Consanguinity	TT*				CC				CT			
	NSOFC	CL/P	CP	Control	NSOFC	CL/P	CP	Control	NSOFC	CL/P	CP	Control
Yes (%)	56 (60.9)	42 (60)	14 (63.6)	77 (55.8)	7 (70)	6 (66.7)	1 (100)	3 (100)	23 (45.1)	17 (43.6)	6 (50)	12 (46.2)
No (%)	36 (39.1)	28 (40)	8 (36.4)	61 (54.2)	3 (30)	3 (33.3)	0	0	28 (54.9)	22 (56.4)	6 (50)	14 (55.8)
Total (100%)	92	70	22	138	10	9	1	3	51	39	12	26
OR (95% CI)	1.23 (0.72-2.11)	1.9 (0.66-2.13)	1.3 (0.5-3.5)		a	a	a		1 (0.39-2.57)	0.9 (0.33-2.44)	1.17 (0.3-4.6)	
X (df), P-value		1.17 (3), 0.77				1.33 (1), 0.514				0.41 (2), 0.813		

* Homozygous common allele genotype

a. Not possible to analyse because the groups contain zero values

A34: Distribution of infant *VAX1* rs7078160 genotypes in cases and controls according to parental consanguinity.

Consanguinity	GG* (%)	AA (%)	AG (%)	Total (%)
Yes	152 (59)	8 (61.5)	17 (39.5)	177 (56.5)
No	105(41)	5(38.5)	26 (60.5)	136 (43.5)
Total	257(100)	13 (100)	43(100)	313(100)

X^2=5.83, df=2, P= 0.054

* Homozygous common allele genotype is the reference

A35: Comparison between case and control infant *VAX1* r rs7078160 genotypes and their relationship to parental consanguinity.

Consanguinity	**GG***				**AA**				**AG**			
	NSOFC	CL/P	CP	Control	NSOFC	CL/P	CP	Control	NSOFC	CL/P	CP	Control
Yes (%)	73 (62)	53 (60.9)	20 (66.7)	79 (56.4)	6 (54.5)	6 (54.4)	0	2 (100)	7 (33.3)	6 (33.3)	1 (33.3)	10 (45.5)
No (%)	44 (38)	34 (39.1)	10 (33.3)	61 (43.6)	5 (45.5)	5 (45.5)	0	0	14 (66.7)	12 (66.7)	2 (66.7)	12 (54.5)
Total (100%)	117	87	30	140	11	11	0	2	21	18	3	22
OR (95% CI)	1.26 (0.77-2.07)	1.2 (0.7-2.08)	1.29 (0.5-2.84)		a	a	a		0.6 (0.17-2.06)	0.6 (0.16-2.18)	0.6 (0.04-7.63)	
X^2(df), P-value		1.24 (2), 0.537				1.48 (1), 0.224				0.66 (2), 0.719		

* Homozygous common allele genotype

a. Not possible to analyse because the groups contain zero values

A36: Distribution of infant *VAX1* rs7078160 alleles in cases and controls with consanguineous parents.

Allele type	NSOFC (%)	CL/P (%)	CP (%)	Control (%)
G*	153 (89)	112 (86.6)	41 (97.7)	168 (92.3)
A	19 (11)	18 (13.4)	1 (2.3)	14 (7.7)
Total	172 (100)	134 (100)	42 (100)	182 (100)
P-value OR (95% CI)	0.28 1.49 (0.72 - 3.07)	0.081 1.93 (0.92 - 4.04)	0.29 0.29 (0.04 – 2.29)	

X^2=6.11, df=2, P= 0.047**

* Common allele is the reference

A37: Maternal infant gene-gene interaction using log-linear model

Table1: Interaction between the maternal and infant genotype variants among NSOFC for IRF6 rs2013162

Mother gene variant	Infant gene variant	P value	95% Confidence Interval	
			Lower Bound	Upper Bound
CC	CC	0.215	-.150-	.669
	AA	0.000**	-4.925-	-1.666-
	CA	0.067	-.958-	.033
AA	CC	0.000**	-3.171-	-1.223-
	AA	0.000**	-2.887-	-1.106-
	CA	0.000**	-3.541-	-1.357-
CA	CC	0.151	-.831-	.128
	AA	0.000**	-2.887-	-1.106-
	CA	a	a	a

a. This parameter is set to zero because it is redundant.
Goodness of fit test was zero.

Table 2: Interaction between the maternal and infant genotype variants among NSOFC for IRF6 rs2235375 gene

Mother gene variant	Infant gene variant	P value	95 % CI	
			Lower Bound	Upper Bound
CC	GG	0.001**	-2.525	-0.694
	CC	0.187	-.986	0.193
	CG	0.021**	-1.457	-0.120
GG	GG	0.029**	0.054	0.997
	CC	0.001**	-4.552	-1.266
	CG	0.393	-0.810	0.318
CG	GG	0.678	-0.660	0.429
	CC	0.071	-1.196	0.049
	CG	a	a	a

.a. This parameter is set to zero because it is redundant.
Goodness of fit test was zero.

Table 3: Interaction between the maternal and infant genotype variants among NSOFC for VAX1 rs4752028 SNP

Mother genotype	Infant genotype	P value	CI	
			Upper level	Lower level
CC	CC	0.000**	3.273	3.922
	TT	0.000**	-3.072	-1.114
	CT	0.000**	-4.825	-1.559
TT	CC	0.000**	-2.560	-0.891
	TT	0.003**	-7.081	-1.500
	CT	0.000**	0.500	10.271
CT	CC	0.005**	-1.451-	-0.262
	TT	0.000**	-2.789-	-0.996
	CT	0.000**	-2.368-	-0.797

Goodness of fit test was zero.

Table 4: Interaction between the maternal and infant genotype variants among NSOFC for VAX1 rs7078160

Mother genotype	Infant genotype	P value	CI	
			Upper level	Lower level
GG	AA	0.219	-1.678-	0.385
	AG	0.000**	1.721	2.987
	AA	1.000	-0.855-	0.855
AA	AA	0.075	-2.308-	0.111
	AG	0.219	-1.678-	0.385
	AA	0.075	-2.308-	0.111
AG	AA	0.133	-1.952-	0.257
	AG	0.426	-0.471-	1.117
	AA	a	a	a

a. This parameter is set to zero because it is redundant.
Goodness of fit test was zero.

Goodness of fit test was zero.

A**3**8: Relationship between maternal IRF6 rs2013162 variants and different environmental factors, including: maternal illness, medication, stress, supplements, paternal smoking and maternal domestic exposure using case-only study design**.**

Maternal rs2013162		**CC* (%)**	**AA (%)**	**CA (%)**
Maternal medication use and illness				
Antibiotic pre-gestation X^2=2.88, df=2, P= 0.237 **N=163**	Yes	13 (17.3)	0 (0)	9 (11.7)
	No	62(82.7)	11 (100)	68 (88.3)
	P value OR (95% CI)		0.277 a	0.325 0.63 (0.25-1.58)
Antibiotic at 1st trimester X^2=1.99, df=2, P= 0.369 **N=136**	Yes	9 (12.0)	1 (9.1)	15 (19.5)
	No	66 (88.0)	10 (90.9)	62 (80.5)
	P value OR (95% CI)		0.779 0.73 (0.08-6.43)	0.21 1.77 (0.72-4.35)
Antipyretic medication pre-gestation X^2=3.46, df=2, P= 0.177 **N=164**	Yes	6 (7.9)	2 (18.2)	3 (3.9)
	No	70 (92.1)	9 (81.8)	74 (96.1)
	P value OR (95% CI)		0.284 2.59 (0.45-14.84)	0.303 0.47 (0.11-1.96)
Antipyretic medication 1st trimester X^2=7.67, df=2, P= 0.022** **N=162**	Yes	4 (5.3)	3 (33.3)	9 (11.7)
	No	72 (94.7)	6 (66.7)	68 (88.3)
	P value OR (95% CI)		0.012** 9 (1.62-49.91)	0.164 2.38(0.7-8.1)
Anti-emetic medication pre-gestation X^2=1.18, df=2, P= 0.554 **N=163**	Yes	1 (1.3)	0 (0)	0 (0)
	No	74 (98.7)	11 (100)	77 (100)
	P value OR (95% CI)		0.644 a	0.488 a
Anti-emetic medication 1st trimester X^2=0.83, df=2, P= 0.659 **N=163**	Yes	9 (12.0)	2 (18.2)	13 (16.9)
	No	66 (88.0)	9 (81.8)	64 (83.1)
	P value OR (95% CI)		0.57 1.63 (0.3-8.77)	0.39 1.49 (0.6-3.73)
Contraceptives pre-gestation X^2=1.73, df=2, P= 0.421 **N=161**	Yes	8 (10.7)	0 (0)	5 (6.5)
	No	67 (89.3)	9 (100)	72 (93.5)
	P value OR (95% CI)		0.56 a	0.362 0.58 (0.18-1.87)
Contraceptives 1st trimester X^2=0.56, df=2, P= 0.757 **N=163**	Yes	1 (1.3)	0 (0)	2 (2.6)
	No	74 (98.7)	11 (100)	75 (97.4)
	P value OR (95% CI)		0.644 a	0.582 1.97 (018-22.23)
Illness pre-gestation X^2=2.24, df=2, P= 0.326 **N**=163	Yes	23 (30.7)	1 (9.1)	21 (27.3)
	No	52 (69.3)	10 (90.9)	56 (72.7)
	P value		0.168	0.64

	OR (95% CI)		0.23 (0.03-1.87)	0.85 (0.42-1.71)
Illness 1st trimester X^2=1.37, df=2, P= 0.505 **N=163**	Yes	25 (33.3)	5 (45.5)	32 (51.6)
	No	50 (66.7)	6 (54.5)	45 (58.4)
	P value OR (95% CI)		0.434 1.67 (0.46-6)	0.3 1.42 (0.73-2.75)
Common cold/flu pre-gestation X^2=5.25, df=2, P= 0.073 **N=145**	Yes	20 (26.7)	0 (0)	13 (16.9)
	No	55 (73.3)	11 (100)	46 (83.1)
	P value OR (95% CI)		0.145 a	0.537 0.78 (0.35-1.73)
Common cold/flu 1st trimester X^2=6, df=2, P= **N=163**	Yes	16 (21.3)	1 (9.1)	19 (24.7)
	No	59 (78.7)	10 (90.9)	58 (75.3)
	P value OR (95% CI)		0.358 0.37 (0.04-3.1)	0.625 1.53 (0.74-3.16)
Fever pre-gestation X^2=7.37, df=2, P= 0.025** **N=163**	Yes	13 (17.3)	0 (0)	4 (5.2)
	No	62 (82.7)	11 (100)	73 (94.8)
	P value OR (95% CI)		0.277 a	0.025** 0.26 (0.08-0.84)
Fever 1st trimester X^2=4.37, df=2, P= 0.112 **N=163**	Yes	8 (10.7)	0 (0)	15 (19.5)
	No	67 (89.3)	11 (100)	62 (80.5)
	P value OR (95% CI)		0.475 a	0.135 2.03 (0.8-5.11)
Urinary tract infection pre-gestation X^2=45, df=2, P= 0.797 **N=162**	Yes	3 (4.0)	0 (0)	3 (3.9)
	No	72 (96.0)	11 (100)	73 (96.1)
	P value OR (95% CI)		0.946 a	0.987 0.99 (0.19-5.05)
Urinary tract infection 1st trimester X^2=0.61, df=2, P= 0.736 **N=162**	Yes	3 (4.0)	0 (0)	2 (2.6)
	No	72 (96.0)	11 (100)	74 (97.4)
	P value OR (95% CI)		0.946 a	0.641 0.65 (0.11-34)
High blood pressure pre-gestation X^2=3.59, df=2, P= 0.166 **N=163**	Yes	3 (4.0)	0 (0)	0 (0)
	No	72 (96.0)	11 (100)	77 (100)
	P value OR (95% CI)		0.946 a	0.186 a
High blood pressure 1st trimester X^2=4.81, df=2, P= 0.09 **N=163**	Yes	4 (5.3)	0 (0)	0 (0)
	No	71 (94.7)	11 (100)	77 (100)
	P value OR (95% CI)		0.808 a	0.129 a
Diabetes pre-gestation a **N=163**	Yes	0 (0)	0 (0)	0 (0)
	No	75 (100)	11 (100)	77 (100)
	P value OR (95% CI)		a	a
Diabetes 1st trimester X^2=1.34, df=2, P= 0.51 **N=163**	Yes	1 (1.3)	0 (0)	3 (3.9)
	No	74 (98.7)	11 (100)	74 (96.1)
	P value OR (95% CI)		0.644 a	0.346 3 (0.31-29.51)
Asthma pre-gestation	Yes	2 (2.7)	0 (0)	1 (1.3)

X^2=0.6, df=2, P= 0.74 **N=162**	No	73 (97.3)	11 (100)	75 (98.7)
	P value OR (95% CI)		0.953 a	0.56 2.9 (0.12-72.9)
Asthma 1st trimester X^2=3.55, df=2, P= 0.17 **N=162**	Yes	3 (4.0)	0 (0)	0 (0)
	No	72 (96.0)	11 (100)	76 (100)
	P value OR (95% CI)		0.946 a	0.189 a
Convulsions pre-gestation *a* **N=163**	Yes	0 (0)	0 (0)	0(0)
	No	75 (100)	11 (100)	77 (100)
	P value OR (95% CI)		a	a
Convulsions 1st trimester a **N=163**	Yes	0 (0)	0 (0)	0(0)
	No	75 (100)	11 (100)	77 (100)
	P value OR (95% CI)		a	a
Vaginal bleeding X^2=2.55, df=2, P= 0.28 **N=162**	Yes	3 (4.0)	0 (0)	7 (9.2)
	No	72 (96.0)	11 (100)	69 (90.8)
	P value OR (95% CI)		0.946 a	0.21 2.43 (0.61-9.8)
Maternal exposure to X-ray 1st trimester X^2=1.22, df=2, P= 0.544 **N=145**	Yes	23 (31.1)	1 (9.1)	3 (4.9)
	No	46 (62.2)	10 (90.9)	62 (95.4)
	P value OR (95% CI)		0.136 0.2 (0.02-1.66)	<0.001** 0.09 (0.03-0.34)
Maternal supplement use				
Folic acid pre-gestation X^2=8.24, df=2,P= 0.016** **N=164**	Yes	2 (2.6)	2 (18.2)	12 (15.6)
	No	74 (97.4)	9 (81.8)	65 (84.4)
	P value OR (95% CI)		0.047** 8.22 (1.03-65.72)	0.014** 6.83 (1.47-31.66)
Folic acid 1st trimester X^2=3.17, df=2,P= 0205 **N=164**	Yes	43 (56.6)	4 (36.4)	49 (63.6)
	No	33 (43.4)	7 (63.6)	28 (36.4)
	P value OR (95% CI)		0.217 0.44 (0.12-1.62)	0.373 1.34 (0.7-2.57)
Multivitamins pre-gestation X^2=3.01, df=2,P= 0.222 **N=164**	Yes	4 (5.3)	2 (18.2)	4 (5.2)
	No	72 (94.7)	9 (81.8)	73 (94.8)
	P value OR (95% CI)		0.138 4 (0.64-25.02)	0.985 0.99 (0.24-4.1)
Multivitamins 1st trimester X^2=4.65, df=2,P= 0.098 **N=163**	Yes	14 (18.7)	5 (45.5)	14 (18.2)
	No	61 (81.3)	6 (54.5)	63 (81.8)
	P value OR (95% CI)		3.63 (0.97-13.61)	0.97 (0.43-2.2)
Iron pre-gestation X^2=1.79, df=2,P= 0.408 **N=163**	Yes	6 (8.0)	2 (18.2)	5 (6.5)
	No	69 (92.0)	9 (81.8)	72 (93.5)
	P value OR (95% CI)		0.292 2.56 (0.45-14.63)	0.721 0.8 (0.23-2.74)

Iron 1st trimester X^2=5, df=2,P= 0.082 **N=163**	Yes	29 (38.7)	1 (9.1)	21 (27.3)
	No	46 (61.3)	10 (90.9)	56 (72.7)
	P value OR (95% CI)		0.087 0.16 (0.02-1.3)	0.137 0.59 (0.3-1.18)
Calcium 1st trimester X^2=1.23, df=2,P= 0.54 **N=163**	Yes	5 (6.7)	0 (0)	3 (3.9)
	No	70 (93.36)	11 (100)	74 (96.1)
	P value OR (95% CI)		0.699 a	0.45 0.57 (0.13-2.46)
Smoking				
Maternal smoking X^2=6, df=2, P= 0.021** **N=162**	Yes	3 (4.0)	2 (18.2)	1 (1.3)
	No	72 (96.0)	9 (81.8)	75 (98.7)
	P value OR (95% CI)		0.087 5.33 (0.78-36.33)	0.329 0.32 (0.03-3.15)
Paternal smoking X^2=0.63, df=2, P= 0.731 **N=162**	Yes	25 (33.3)	5 (45.5)	27 (35.5)
	No	50 (66.7)	6 (54.5)	49 (64.5)
	P value OR (95% CI)		0.434 1.67 (0.46-6)	0.777 1.1 (0.56-2.16)
Paternal tobacco X^2=2.27, df=2, P= 0.322 **N=162**	Yes	18 (24.0)	5 (54.5)	20 (26.3)
	No	57 (76.0)	6 (54.5)	56 (73.7)
	P value OR (95% CI)		0.143 2.64 (0.72-9.68)	0.743 1.13 (0.54-2.36)
Paternal waterpipe X^2=1.96, df=2, P= 0.376 **N=162**	Yes	10 (13.3)	3 (27.3)	9 (11.8)
	No	65 (86.7)	8 (72.7)	67 (88.2)
	P value OR (95% CI)		0.24 2.44 (0.55-10.76)	0.783 0.87 (0.33-2.29)
Maternal passive smoking X^2=5.29, df=2, P= 0.071 **N=162**	Yes	16 (21.3)	5 (45.5)	12 (15.8)
	No	59 (78.7)	6 (54.5)	64 (84.2)
	P value OR (95% CI)		0.09 3.07 (0.83-11.38)	0.382 0.69 (0.3-1.58)
Maternal stress				
Family problems X^2=3.41, df=2, P= 0.182 **N=162**	Yes	26 (34.7)	7 (63.6)	30 (39.5)
	No	49 (65.3)	4 (36.4)	46 (60.5)
	P value OR (95% CI)		0.076 3.3 (0.88-12.31)	0.541 1.23 (0.63-2.38)
Mother complains of being under stress X^2=1.71, df=2, P= 0.425 **N=162**	Yes	32 (42.7)	7 (63.6)	34 (44.7)
	No	43 (57.3)	4 (36.4)	42 (55.3)
	P value OR (95% CI)		0.201 2.35 (0.63-8.72)	0.798 1.08 (0.57-2.07)
Depression pre-gestation X^2=3.41, df=2, P= 0.181 **N=163**	Yes	0 (0)	0 (0)	3 (3.9)
	No	75 (100)	11 (100)	74(96.1)
	P value OR (95% CI)		a	a

Depression 1st trimester X^2=5.68, df=2, P= 0.059 **N=163**	Yes	2 (2.7)	2 (18.2)	3 (3.9)
	No	73 (97.3)	9 (81.8)	74(96.1)
	P value OR (95% CI)		0.048** 8.11 (1.01-64.84)	0.673 1.48 (0.24-9.12)
Severe morning sickness X^2=1.66, df=2, P= 0.44 **N=162**	Yes	10 (13.3)	0 (0)	10 (13.2)
	No	65 (86.7)	11 (100)	66 (86.8)
	P value OR (95% CI)		0.379 a	0.975 0.98 (0.38-2.52)
Threatened abortion X^2=2.96, df=2, P= 0.23 **N=162**	Yes	2 (2.7)	1 (9.1)	7 (9.2)
	No	73(97.3)	10 (90.9)	69 (90.8)
	P value OR (95% CI)		0.308 3.65 (0.3-44.02)	0.11 3.7 (0.74-18.44)
Abdominal pain pre-gestation X^2=1.17, df=2, P= 0.558 **N=162**	Yes	2 (2.7)	0 (0)	4 (5.3)
	No	73 (97.3)	11 (100)	72 (94.7)
	P value OR (95% CI)		0.877 a	0.423 2.03 (0.36-11.42)
Abdominal pain 1st trimester X^2=7.86, df=2, P= 0.02** **N=162**	Yes	6 (8.0)	4 (36.4)	8 (10.5)
	No	69 (92.0)	7 (63.6)	68 (89.5)
	P value OR (95% CI)		0.013** 6.57 (1.49-29.01)	0.594 1.35 (0.45-4.1)
Maternal domestic environmental exposure				
Exposure to chemicals pre-gestation X^2=3.77, df=2, P= 0.152	Yes	25 (33.3)	6 (54.5)	20 (26.3)
	No	50 (66.7)	5 (45.5)	56 (73.7)
	P value OR (95% CI)		0.18 2.4 (0.67-8.63)	0.347 0.71 (0.35-1.44)
Exposure to chemicals 1st trimester X^2=4.97, df=2, P= 0.083 **N=160**	Yes	25 (33.3)	6 (54.5)	18 (23.7)
	No	50 (66.7)	5 (45.5)	56 (76.3)
	P value OR (95% CI)		0.18 2.4 (0.67-8.63)	0.226 0.64 (0.31-1.32)
Exposure to solvents pre-gestation X^2=1.83, df=2, P= 0.401 **N=162**	Yes	12 (16.0)	2 (18.2)	7 (9.2)
	No	63 (84.0)	9 (81.8)	69 (90.8)
	P value OR (95% CI)		0.855 1.17 (0.22-6.09)	0.214 0.53 (0.2-1.44)
Exposure to solvents 1st trimester X^2=4.07, df=2, P= 0.13 **N=162**	Yes	11 (14.7)	0 (0)	5 (6.6)
	No	64 (85.3)	11 (100)	71 (93.4)
	P value OR (95% CI)		0.34 a	0.115 0.41 (0.14-1.24)
Exposure to pesticides pre-gestation X^2=0.34, df=2, P= 0.84 **N=162**	Yes	11 (14.7)	1 (9.1)	12 (15.8)
	No	64 (85.3)	10 (90.9)	64 (84.2)
	P value OR (95% CI)		0.622 0.58 (0.07-5)	0.848 1.09 (0.45-2.65)

Exposure to pesticides 1st trimester X^2=0.86, df=2, P= 0.652 **N=162**	Yes	12 (16.0)	3 (27.3)	13 (17.1)
	No	63 (84.0)	8 (72.7)	63 (82.9)
	P value OR (95% CI)		0.364 1.97 (0.46-8.51)	0.855 1.08 (0.46-2.56)
Exposure to incense pre-gestation X^2=2.01, df=2, P= 0.365 **N=162**	Yes	31 (41.3)	4 (36.4)	23 (30.3)
	No	44 (58.7)	7 (63.6)	53 (69.7)
	P value OR (95% CI)		0.754 0.81 (0.22-3.01)	0.157 0.62 (0.31-1.21)
Exposure to incense 1st trimester X^2=1.17, df=2, P= 0.558 **N=162**	Yes	30 (40.0)	4 (36.4)	24 (31.6)
	No	45 (60.0)	7 (63.6)	52 (68.4)
	P value OR (95% CI)		0.81 0.86 (0.23-3.18)	0.281 0.69 (0.35-1.35)
Type of maternal drinking water X^2=7.96, df=2, P= 0.241 **N=147**	Tap	23 (31.1)	1 (9.1)	12 (19.4)
	Bottled	46 (62.2)	10 (90.9)	45 (72.6)
	P value OR (95% CI)		0.136 0.2 (0.02-1.66)	0.128 0.53 (0.24-1.2)
	Well	5 (6.8)	0	3 (4.8)
	P value OR (95% CI)		0.84 a	0.863 0.87 (0.18-4.27)
	Zamzam	0	0	2 (3.2)
	P value OR (95% CI)	a	a	0.16 a
Consanguinity X^2=7.96, df=2, P= 0.241 **N=157**	Yes	40 (57.1)	6 (50)	42 (56)
	No	30 (42.9)	6 (50)	33 (44)
	P value OR (95% CI)		0.646 0.75 (0.22-2.56)	0.89 0.95 (0.49-1.84)
Family history X^2=0.37, df=2, P= 0.985 **N=162**	Yes	30 (40.0)	5 (54.5)	34 (44.7)
	No	45 (60.0)	6 (54.5)	42 (55.3)
	P value OR (95% CI)		0.731 1.25 (0.35-4.47)	0.556 1.2 (0.64-2.32)

*Homozygous common allele genotype.

**The Chi-square statistic is significant at the 0.05 level.

[a] Could not analyse because the groups contained zero values.
If one of the cells contained a zero value, the OR and 95% CI were not calculated.

A39: Relationship between Maternal IRF6 rs2013162 allele and different environmental factors, including: maternal illness, medication, stress, supplements, paternal smoking and maternal domestic exposure using case-only study design.

Maternal *IRF6* rs2013162		**C* (%)**	**A (%)**
Maternal medication use and illness			
Antibiotic pre-gestation **N=326**	Yes	35 (15.4)	9 (9.1)
	No	192 (84.6)	90 (90.9)
	X^2 (df), P value OR (95% CI)	2.36 (1), 0.129 0.55 (0.25-1.19)	
Antibiotic at 1st trimester **N=279**	Yes	33 (18.3)	17 (17.2)
	No	147 (81.7)	82 (82.8)
	X^2 (df), P value OR (95% CI)	0.06 (1), 0.809 0.92 (0.48-1.76)	
Antipyretic medication pre-gestation **N=328**	Yes	15 (6.6)	7 (7.1)
	No	214 (93.4)	92 (92.9)
	X^2 (df), P value OR (95% CI)	0.03 (1), 0.863 1.09 (0.43-2.75)	
Antipyretic medication 1st trimester **N=324**	Yes	17 (7.4)	15 (15.8)
	No	212 (92.6)	80 (84.2)
	X^2 (df), P value OR (95% CI)	5.28 (1), 0.025** 2.34 (1.12-4.9)	
Anti-emetic medication pre-gestation **N=326**	Yes	2 (0.9)	0
	No	225 (99.1)	99 (100)
	X^2 (df), P value	0.88 (1), 0.35	
Anti-emetic medication 1st trimester **N=326**	Yes	31 (13.7)	17 (17.2)
	No	196 (86.3)	82 (82.8)
	X^2 (df), P value OR (95% CI)	0.68 (1), 0.411 1.31 (0.69-2.5)	
Contraceptives pre-gestation **N=322**	Yes	21 (9.3)	5 (5.3)
	No	206 (90.7)	90 (94.7)
	X^2 (df), P value OR (95% CI)	1.44 (1), 0.23 0.55 (0.2-1.49)	
Contraceptives 1st trimester **N=326**	Yes	4 (1.8)	2 (2)
	No	223 (98.2)	97 (98)
	X^2 (df), P value OR (95% CI)	0.03 (1), 0.87 1.15 (0.21-6.38)	
Illness pre-gestation **N=326**	Yes	67 (29.5)	23 (23.2)
	No	160 (70.5)	76 (76.8)
	X^2 (df), P value OR (95% CI)	1.36 (1), 0.244 0.72 (0.42-1.25)	
Illness 1st trimester	Yes	82 (26.1)	42 (42.4)
	No	145 (63.9)	57 (57.6)

N=326	X^2 (df), P value OR (95% CI)	1.16 (1), 0.282 1.3 (0.8-2.11)	
Common cold/flu pre-gestation **N=290**	Yes	53 (25.4)	13 (16.1)
	No	156 (74.6)	68 (83.9)
	X^2 (df), P value OR (95% CI)	2.88 (1), 0.90 0.56 (0.29-1.1)	
Common cold/flu 1st trimester **N=326**	Yes	51 (22.5)	21 (21.2)
	No	176 (77.5)	78 (78.8)
	X^2 (df), P value OR (95% CI)	0.06 (1), 0.802 0.93 (0.52-1.65)	
Fever pre-gestation **N=326**	Yes	30 (13.2)	4 (4)
	No	197 (86.8)	95 (96)
	X^2 (df), P value OR (95% CI)	6.21 (1), 0.019** 0.28 (0.09-0.81)	
Fever 1st trimester **N=326**	Yes	31 (13.7)	15 (15.2)
	No	196 (86.3)	84 (84.8)
	X^2 (df), P value OR (95% CI)	0.13 (1), 0.72 1.13 (0.58-2.2)	
Urinary tract infection pre-gestation **N=324**	Yes	9 (4)	3 (3.1)
	No	217 (96)	95 (96.9)
	X^2 (df), P value OR (95% CI)	0.16 (1), 0.69 0.76 (0.2-2.88)	
Urinary tract infection 1st trimester **N=324**	Yes	8 (3.5)	2 (2)
	No	218 (96.5)	96 (98)
	X^2 (df), P value OR (95% CI)	0.51 (1) 0.479 0.57 (0.12-2.72)	
High blood pressure pre-gestation **N=326**	Yes	6 (2.6)	0
	No	221 (97.4)	99 (100)
	X^2 (df), P value	2.67 (1), 0.10	
High blood pressure 1st trimester **N=326**	Yes	8 (3.5)	0
	No	219 (96.5)	99 (100)
	X^2 (df), P value	3.57 (1), 0.06	
Diabetes pre-gestation **N=290**	Yes	0	0
	No	191 (100)	99 (100)
	X^2 (df), P value	a	
Diabetes 1st trimester **N=326**	Yes	5 (2.2)	3 (3)
	No	222 (97.8)	96 (97)
	X^2 (df), P value OR (95% CI)	0.20 (1), 0.658 1.39 (0.33-5.92)	
Asthma pre-gestation **N=324**	Yes	5 (2.2)	1 (1)
	No	221 (97.8)	97 (99)
	X^2 (df), P value OR (95% CI)	0.53 (1), 0.476 0.46 (0.05-3.95)	
Asthma 1st trimester **N=324**	Yes	6 (2.7)	0
	No	220 (97.3)	98 (100)
	X^2 (df), P value	2.65 (1), 0.10	
Convulsions pre-gestation **N=326**	Yes	0	0
	No	227 (100)	99 (100)

	X^2 (df), P value	a	
Convulsions 1st trimester **N=326**	Yes	0	0
	No	227 (100)	99 (100)
	X^2 (df), P value	a	
Vaginal bleeding **N=324**	Yes	13 (5.8)	7 (7.1)
	No	213 (94.2)	91 (92.9)
	X^2 (df), P value OR (95% CI)	0.23 (1), 0.634 1.26 (0.49-3.26)	
Maternal supplement use			
Folic acid pre-gestation **N=328**	Yes	16 (7)	16 (16.2)
	No	213 (93)	83 (83.8)
	X^2 (df), P value OR (95% CI)	6.61 (1), 0.012** 2.57 (1.23-5.37)	
Folic acid 1st trimester **N=328**	Yes	135 (59)	57 (57.6)
	No	94 (41)	42 (42.4)
	X^2 (df), P value OR (95% CI)	0.05 (1), 0.816 0.95 (0.59-1.52)	
Multivitamins pre-gestation **N=328**	Yes	12 (5.2)	8 (8.1)
	No	217 (94.8)	91 (91.9)
	X^2 (df), P value OR (95% CI)	0.97 (1), 0.327 1.59 (0.63-4.02)	
Multivitamins 1st trimester **N=326**	Yes	42 (18.5)	24 (24.2)
	No	185 (81.5)	75 (75.8)
	X^2 (df), P value OR (95% CI)	1.41 (1), 0.24 1.41 (0.8-2.49)	
Iron pre-gestation **N=326**	Yes	17 (7.5)	9 (9)
	No	210 (92.5)	90 (90)
	X^2 (df), P value OR (95% CI)	0.24 (1), 0.62 1.24 (0.53-2.88)	
Iron 1st trimester **N=326**	Yes	79 (34.8)	23 (23.2)
	No	148 (65.2)	76 (76.8)
	X^2 (df), P value OR (95% CI)	4.29 (1), 0.04** 0.57 (0.33-0.97)	
Calcium 1st trimester **N=326**	Yes	13 (5.7)	3 (3)
	No	214 (94.3)	96 (97)
	X^2 (df), P value OR (95% CI)	1.07 (1), 0.30 0.51 (0.14-1.85)	
Smoking			
Maternal smoking **N=324**	Yes	7 (3.1)	5 (5.1)
	No	219 (96.9)	93 (94.9)
	X^2 (df), P value OR (95% CI)	0.77 (1), 0.38 1.68 (0.52-5.44)	
Paternal smoking **N=324**	Yes	77 (34.1)	37 (37.8)
	No	149 (65.9)	61 (62.2)
	X^2 (df), P value OR (95% CI)	0.41 (1), 0.52 1.17 (0.72-1.92)	
Paternal tobacco **N=310**	Yes	56 (25.6)	30 (33)
	No	163 (74.4)	61 (67)

	X^2 (df), P value OR (95% CI)	1.75 (1), 0.19 1.43 (0.84-2.44)	
Paternal waterpipe **N=324**	Yes	29 (12.8)	15 (15.3)
	No	197 (87.1)	83 (84.7)
	X^2 (df), P value OR (95% CI)	0.36 (1), 0.55 1.22 (0.63-2.41)	
Maternal passive smoking **N=324**	Yes	44 (19.5)	22 (22.5)
	No	182 (80.5)	76 (77.5)
	X^2 (df), P value OR (95% CI)	0.37 (1) 0.54 1.2 (0.67-2.13)	
Maternal stress			
Family problems **N=324**	Yes	82 (36.3)	44 (44.9)
	No	144 (63.7)	54 (55.1)
	X^2 (df), P value OR (95% CI)	2.13 (1) 0.14 1.43 (0.88-2.32)	
Mother complains of being under stress **N=324**	Yes	98 (43.4)	48 (49)
	No	128 (56.6)	50 (51)
	X^2 (df), P value OR (95% CI)	0.87 (1), 0.35 1.25 (0.78-2.02)	
Depression pre-gestation **N=326**	Yes	3 (1.3)	3 (3)
	No	224 (98.7)	96 (97)
	X^2 (df), P value OR (95% CI)	1.11 (1), 0.29 2.33 (0.46-11.77)	
Depression 1st trimester **N=326**	Yes	7 (3.1)	7 (7.1)
	No	220 (96.9)	92 (92.9)
	X^2 (df), P value OR (95% CI)	2.67 (1), 0.10 2.39 (0.82-7.01)	
Severe morning sickness **N=324**	Yes	30 (12.3)	10 (10.2)
	No	196 (86.7)	88 (88.8)
	X^2 (df), P value OR (95% CI)	0.60 (1) 0.441 0.74 (0.35-1.59)	
Threatened abortion **N=344**	Yes	11 (4.9)	9 (7.6)
	No	215 (95.1)	109 (92.4)
	X^2 (df), P value OR (95% CI)	1.08 (1) 0.302 1.61 (0.65-4.01)	
Abdominal pain pre-gestation **N=324**	Yes	8 (3.5)	4 (4.1)
	No	218 (96.5)	94 (95.9)
	X^2 (df), P value OR (95% CI)	0.06 (1) 0.81 1.16 (0.34-3.94)	
Abdominal pain 1st trimester **N=324**	Yes	20 (8.9)	16 (16.3)
	No	206 (91.1)	82 (83.7)
	X^2 (df), P value OR (95% CI)	3.87 (1) 0.053 2 (0.99-4.07)	
Maternal domestic environmental exposure			
Exposure to chemicals pre-gestation **N=324**	Yes	70 (31)	32 (32.7)
	No	156 (69)	66 (67.3)
	X^2 (df), P value OR (95% CI)	0.09 (1) 0.76 1.08 (0.65-1.8)	

Exposure to chemicals 1st trimester **N=320**	Yes	68 (30.4)	30 (31.3)
	No	156 (69.6)	66 (68.7)
	X^2 (df), P value OR (95% CI)	0.03 (1) 0.874 1.04 (0.62-1.75)	
Exposure to solvents pre-gestation **N=324**	Yes	31 (13.7)	11 (11.2)
	No	195 (86.3)	87 (88.8)
	X^2 (df), P value OR (95% CI)	0.38 (1) 0.54 0.8 (0.38-1.66)	
Exposure to solvents 1st trimester **N=324**	Yes	27 (12)	5 (5.1)
	No	199 (88)	93 (94.9(
	X^2 (df), P value OR (95% CI)	3.60 (1) 0.066 0.4 (0.15-1.07)	
Exposure to pesticides pre-gestation **N=324**	Yes	34 (15)	14 (14.3)
	No	192 (85)	84 (85.7)
	X^2 (df), P value OR (95% CI)	0.03 (1) 0.86 0.94 (0.48-1.85)	
Exposure to pesticides 1st trimester **N=324**	Yes	37 (16.4)	19 (19.4)
	No	189 (83.6)	79 (80.6)
	X^2 (df), P value OR (95% CI)	0.44 (1) 0.51 1.23 (0.67-2.27)	
Exposure to incense pre-gestation **N=324**	Yes	85 (37.6)	31 (31.6)
	No	141 (62.4)	67 (68.4)
	X^2 (df), P value OR (95% CI)	1.06 (1) 0.303 0.77 (0.36-1.27)	
Exposure to incense 1st trimester **N=324**	Yes	84 (37.2)	32 (32.7)
	No	142 (62.8)	66 (67.3)
	X^2 (df), P value OR (95% CI)	0.61 (1) 0.44 0.82 (0.5-1.35)	
Maternal exposure to X-ray 1st trimester **N=290**	Yes	49 (24.1)	5 (5.8)
	No	154 (75.9)	82 (94.2)
	X^2 (df), P value OR (95% CI)	13.59 (1) <0.001** 0.19 (0.07-0.5)	
Type of maternal drinking water **N=290**	Tap (reference)	58 (27.9)	14 (17.1)
	Bottle	137 (65.9)	65 (79.3)
	X^2 (df), P value OR (95% CI)	4.19 (1) 0.04** 0.51 (0.26-0.98)	
	Well	13 (6.2)	3 (3.6)
	X^2 (df), P value OR (95% CI)	0.004 (1) 0.949 1.05 (0.26-4.18)	
	Zamzam	0	0
	OR (95% CI)	a	
Consanguinity **N=314**	Yes	122 (56.7)	54 (54.6)
	No	93 (43.3)	45 (45.4)
	P value OR (95% CI)	0.13 (1) 0.715 0.91 (0.57-1.48)	
Family history	Yes	94 (41.6)	44 (44.9)
	No	132 (58.4)	54 (55.1)

N=324	X^2 (df), P value OR (95% CI)	0.31 (1) 0.58 1.14 (0.71-1.85)

*Common allele

**Significance level P ≤0.05

[a] Not possible to analyse because the groups contain zero values

If one of the cells contains zero value, the OR and 95% CI are not calculated

A40: Comparison between cases and controls for maternal *IRF6* rs2013162 genotypes in relationship to different environmental factors including: maternal illness, medication, stress, supplements, paternal smoking and maternal domestic exposure.

Maternal *IRF6* rs2013162 genotype		**CC* (%)**		**AA (%)**		**CA (%)**	
Environmental factors		Study	Control	Study	Control	Study	Control
Maternal medication use and illness							
Antibiotic use pre-gestation **Study Group N = 163 Control Group N=175**	Yes	13 (17.3)	3 (3.5)	0 (0)	1 (3.7)	9 (11.7)	4 (6.5)
	No	62(82.7)	83 (96.5)	11 (100)	26 (96.3)	68 (88.3)	58 (93.5)
	X^2 (df), P value OR (95% CI)	8.58 (1), 0.003** 5.8(1.58-21.24)		0.42 (1), 0.518		1.11 (1), 0.292	
Antibiotic use 1st trimester **Study Group N = 163 Control Group N=175**	Yes	9 (12.0)	7 (8.1)	1 (9.1)	2 (7.4)	15 (19.5)	9 (14.5)
	No	66 (88.0)	79 (91.9)	10 (90.9)	25 (29.6)	62 (80.5)	53 (85.5)
	X^2 (df), P value	0.67 (1), 0.414		0.03 (1), 0.861		0.59 (1), 0.441	
Antipyretic medication pre-gestation **Study Group N = 164 Control Group N=175**	Yes	6 (7.9)	10 (11.6)	2 (18.2)	3 (11.1)	3 (3.9)	5 (8.1)
	No	70 (92.1)	76 (88.4)	9 (81.8)	24 (88.9)	74 (96.1)	57 (91.9)
	X^2 (df), P value	0.63 (1), 0.427		0.34 (1), 0.559		1.1 (1), 0.294	
Antipyretic medication 1st trimester **Study Group N = 162 Control Group N=172**	Yes	4 (5.3)	12 (14.1)	3 (33.3)	2 (7.4)	9 (11.7)	11 (18.3)
	No	72 (94.7)	73 (85.9)	6 (66.7)	25 (92.6)	68 (88.3)	49 (81.7)
	X^2 (df), P value	3.51 (1), 0.061		3.79 (1), 0.051		1.19 (1), 0.274	
Anti-emetic medication pre-gestation **Study Group N = 163 Control Group N=175**	Yes	1 (1.3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
	No	74 (98.7)	86 (100)	11 (100)	27 (100)	77 (100)	62 (100)
	X^2 (df), P value	1.15 (1), 0.283		a		a	
Anti-emetic medication 1st trimester **Study Group N = 163 Control Group N=175**	Yes	9 (12.0)	5 (5.8)	2 (18.2)	3 (11.1)	13 (16.9)	6 (9.7)
	No	66 (88.0)	81 (94.2)	9 (81.8)	24 (88.9)	64 (83.1)	56 (90.3)
	X^2 (df), P value	1.93 (1), 0.165		0.34 (1), 0.559		1.51 (1), 0.219	
Contraceptives pre-	Yes	8 (10.7)	6 (7.0)	0 (0)	2 (7.4)	5 (6.5)	6 (9.7)

gestation **Study Group N = 161** **Control Group N=175**	No	67 (89.3)	80 (93.0)	9 (100)	25 (92.6)	72 (93.5)	56 (90.3)
	X^2 (df), P value	0.69 (1), 0.407		0.71 (1), 0.401		0.48 (1), 0.489	
Contraceptives 1st trimester **Study Group N = 163** **Control Group N=175**	Yes	1 (1.3)	1 (1.2)	0 (0)	2 (7.4)	2 (2.6)	2 (3.2)
	No	74 (98.7)	85 (98.8)	11 (100)	25 (92.6)	75 (97.4)	60 (96.8)
	X^2 (df), P value	0.009 (1), 0.922		0.86 (1), 0.354		0.05 (1), 0.826	
Illness pre-gestation **Study Group N = 163** **Control Group N=174**	Yes	23 (30.7)	12 (14.0)	1 (9.1)	2 (7.4)	21 (27.3)	5 (8.2)
	No	52 (69.3)	74 (86.0)	10 (90.9)	25 (92.6)	56 (72.7)	56 (91.8)
	X^2 (df), P value OR (95% CI)	6.59 (1), 0.010** 2.73 (1.25-5.97)		0.03 (1), 0.86		8.1 (1), 0.004** 4.2 (1.48-11.92)	
Illness 1st trimester **Study Group N = 163** **Control Group N=173**	Yes	25 (33.3)	23 (26.7)	5 (45.5)	7 (25.9)	32 (51.6)	17 (28.3)
	No	50 (66.7)	63 (73.3)	6 (54.5)	20 (74.1)	45 (58.4)	43 (71.7)
	X^2 (df), P value	0.83 (1), 0.362		1.38 (1), 0.240		2.56 (1), 0.109	
Common cold/flu pre-gestation **Study Group N = 145** **Control Group N=173**	Yes	20 (26.7)	3 (3.5)	0 (0)	1 (3.7)	13 (16.9)	3 (5.0)
	No	55 (73.3)	83 (96.5)	11 (100)	26 (96.3)	46 (83.1)	57 (95.0)
	X^2 (df), P value OR (95% CI)	17.58 (1), <0.001** 10.06 (2.85-35.48)		0.42 (1), 0.518		04.62 (1), .032** 5.37 (1.44-19.98)	
Common cold/flu 1st trimester **Study Group N = 163** **Control Group N=173**	Yes	16 (21.3)	9 (10.5)	1 (9.1)	3 (11.1)	19 (24.7)	12 (20.0)
	No	59 (78.7)	77 (89.5)	10 (90.9)	24 (88.9)	58 (75.3)	48 (80.0)
	X^2 (df), P value	3.61 (1), 0.058		0.03 (1), 0.854		0.42 (1), 0.516	
Fever pre-gestation **Study Group N = 163** **Control Group N=173**	Yes	13 (17.3)	4 (4.7)	0 (0)	1 (3.7)	4 (5.2)	3 (5.0)
	No	62 (82.7)	82 (95.3)	11 (100)	26 (96.3)	73 (94.8)	57 (95.0)
	X^2 (df), P value OR (95% CI)	6.82 (1), 0.009** 4.3 (1.34-13.82)		0.42 (1), 0.518		0.003 (1), 0.959	
Fever 1st trimester **Study Group N = 163** **Control Group N=173**	Yes	8 (10.7)	7 (8.1)	0 (0)	2 (7.4)	15 (19.5)	6 (10.0)
	No	67 (89.3)	79 (91.9)	11 (100)	25 (92.6)	62 (80.5)	54 (90.0)
	X^2 (df), P value	0.3 (1), 0.582		0.86 (1), 0.354		2.34 (1), 0.126	
Urinary tract infection pre-gestation	Yes	3 (4.0)	1 (1.2)	0 (0)	1 (3.7)	3 (3.9)	2 (3.3)
	No	72 (96.0)	85 (98.8)	11 (100)	26 (96.3)	73 (96.1)	58 (96.7)

Study Group N = 162 Control Group N=173	X^2 (df), P value	1.33 (1), 0.249		0.42 (1), 0.518		0.04 (1), 0.850	
Urinary tract infection 1st trimester	Yes	3 (4.0)	4 (4.7)	0 (0)	1 (3.7)	2 (2.6)	2 (3.3)
	No	72 (96.0)	82 (95.3)	11 (100)	26 (96.3)	74 (97.4)	58 (96.7)
Study Group N = 162 Control Group N=173	X^2 (df), P value	0.04 (1), 0.840		0.42 (1), 0.518		0.06 (1), 0.810	
High blood pressure pre-gestation	Yes	3 (4.0)	1 (1.2)	0 (0)	0 (0)	0 (0)	0 (0)
	No	72 (96.0)	85 (98.8)	11 (100)	27 (100)	77 (100)	60 (100)
Study Group N = 163 Control Group N=173	X^2 (df), P value	1.33 (1), 0.249		a		a	
High blood pressure 1st trimester	Yes	4 (5.3)	1 (1.2)	0 (0)	0 (0)	0 (0)	1 (1.7)
	No	71 (94.7)	85 (98.8)	11 (100)	27 (100)	77 (100)	59 (98.3)
Study Group N = 163 Control Group N=173	X^2 (df), P value	2.32 (1), 0.128		a		1.29 (1) 0.256	
Diabetes pre-gestation	Yes	0 (0)	1 (1.2)	0 (0)	0 (0)	0 (0)	0 (0)
	No	57 (100)	85(98.8)	11 (100)	27 (100)	77 (100)	60 (100)
Study Group N = 145 Control Group N=173	X^2 (df), P value	0.88 (1), 0.349		a		a	
Diabetes 1st trimester	Yes	1 (1.3)	3 (3.5)	0 (0)	0 (0)	3 (3.9)	0 (0)
	No	74 (98.7)	83 (96.5)	11 (100)	27 (100)	74 (96.1)	60 (100)
Study Group N = 163 Control Group N=173	X^2 (df), P value	0.77 (1), 0.381		a		0.122	
Asthma pre-gestation	Yes	2 (2.7)	0 (0)	0 (0)	0 (0)	1 (1.3)	1 (1.7)
	No	73 (97.3)	86 (100)	11 (100)	27 (100)	75 (98.7)	59 (98.3)
Study Group N = 162 Control Group N=173	X^2 (df), P value	2.32 (1), 0.128		a		0.028 (1), 0.866	
Asthma 1st trimester	Yes	3 (4.0)	1 (1.2)	0 (0)	1 (3.7)	0 (0)	2 (3.3)
	No	72 (96.0)	85 (98.8)	11 (100)	26 (96.3)	76 (100)	58 (96.7)
Study Group N = 162 Control Group N=173	X^2 (df), P value	1.33 (1), 0.249		0.42 (1), 0.518		2.57 (1), 0.109	
Convulsions pre-gestation	Yes	0 (0)	0 (0)	0 (0)	0 (0)	0(0)	0 (0)
	No	75 (100)	86 (100)	11 (100)	27 (100)	77 (100)	60 (100)

Study Group N = 163 Control Group N=173	X^2 (df), P value	a		a		a	
Convulsions 1st trimester **Study Group N = 163 Control Group N=173**	Yes	0 (0)	0 (0)	0 (0)	0 (0)	0(0)	0 (0)
	No	75 (100)	86 (100)	11 (100)	27 (100)	77 (100)	60 (100)
	X^2 (df), P value	a		a		a	
Vaginal bleeding **Study Group N = 162 Control Group N=173**	Yes	3 (4.0)	1 (1.2)	0 (0)	1 (3.7)	7 (9.2)	3 (5.0)
	No	72 (96.0)	85 (98.8)	11 (100)	26 (96.3)	69 (90.8)	57 (95.0)
	X^2 (df), P value	1.33 (1), 0.249		0.42 (1), 0.518		0.87 (1), 0.350	
Maternal exposure to X-ray 1st trimester **Study Group N = 164 Control Group N=175**	Yes	4 (5.3)	1 (1.2)	0	1 (3.7)	2 (2.6)	0
	No	72 (94.7)	85 (98.8)	11 (100)	26 (96.3)	75 (97.4)	62 (100)
	X^2 (df), P value	2.27 (1), 0.132		0.42 (1), 0.518		1.63 (1), 0.201	
Maternal supplement use							
Folic acid pre-gestation **Study Group N = 164 Control Group N=175**	Yes	2 (2.6)	5 (5.8)	2 (18.2)	0 (0)	12 (15.6)	12 (19.4)
	No	74 (97.4)	81 (94.2)	9 (81.8)	27 (100)	65 (84.4)	50 (80.6)
	X^2 (df), P value	1 (1), 0.320		5.18 (1), 0.023**		0.34 (1), 0.559	
Folic acid 1st trimester **Study Group N = 164 Control Group N=175**	Yes	43 (56.6)	56 (65.1)	4 (36.4)	23 (85.2)	49 (63.6)	46 (74.2)
	No	33 (43.4)	30 (34.9)	7 (63.6)	4 (14.8)	28 (36.4)	16 (25.8)
	X^2 (df), P value OR (95% CI)	1.24 (1), 0.226		9.06 (1), 0.003** 0.1 (0.02-0.50)		1.77 (1), 0.183	
Multivitamins pre-gestation **Study Group N = 164 Control Group N=175**	Yes	4 (5.3)	2 (2.3)	2 (18.2)	0 (0)	4 (5.2)	2 (3.2)
	No	72 (94.7)	84 (97.7)	9 (81.8)	27 (100)	73 (94.8)	60 (96.8)
	X^2 (df), P value	0.98 (1), 0.323		5.18 (1), 0.023**		0.32 (1),0.570	
Multivitamins 1st trimester **Study Group N = 163 Control Group N=175**	Yes	14 (18.7)	15 (17.4)	5 (45.5)	6 (22.2)	14 (18.2)	11 (17.7)
	No	61 (81.3)	71 (82.6)	6 (54.5)	21 (77.8)	63 (81.8)	51 (82.3)
	X^2 (df), P value	0.04 (1), 0.840		2.05 (1), 0.152		0.005 (1), 0.946	
Iron pre-gestation **Study Group N = 163**	Yes	6 (8.0)	3 (3.5)	2 (18.2)	1 (3.7)	5 (6.5)	6 (9.7)
	No	69 (92.0)	83 (96.5)	9 (81.8)	26 (96.3)	72 (93.5)	56 (90.3)

Control Group N=175	X^2 (df), P value	1.55 (1),0.214		2.25 (1) 0.133		0.48 (1), 0.489	
Iron 1st trimester **Study Group N = 163 Control Group N=175**	Yes	29 (38.7)	28 (32.6)	1 (9.1)	9 (33.3)	21 (27.3)	23 (37.1)
	No	46 (61.3)	58 (67.4)	10 (90.9)	18 (66.7)	56 (72.7)	39 (62.9)
	X^2 (df), P value	0.654 (1), 0.419		2.37 (1), 0.124		1.53 (1), 0.216	
Calcium 1st trimester **Study Group N = 163 Control Group N=175**	Yes	5 (6.7)	10 (11.6)	0 (0)	4 (14.8)	3 (3.9)	6 (9.7)
	No	70 (93.36)	76 (88.4)	11 (100)	23 (85.2)	74 (96.1)	56 (90.3)
	X^2 (df), P value	1.17 (1), 0.280		1.18 (1), 0.177		1.9 (1), 0.169	
Smoking							
Maternal smoking **Study Group N = 162 Control Group N=175**	Yes	3 (4.0)	6 (7.0)	2 (18.2)	1 (3.7)	1 (1.3)	1 (1.6)
	No	72 (96.0)	80 (93.0)	9 (81.8)	26 (96.3)	75 (98.7)	61 (98.4)
	X^2 (df), P value	0.67 (1), 0.412		2.25 (1), 0.133		0.02 (1), 0.884	
Paternal smoking **Study Group N = 162 Control Group N=175**	Yes	25 (33.3)	30 (34.9)	5 (45.5)	8 (29.6)	27 (35.5)	22 (35.5)
	No	50 (66.7)	56 (65.1)	6 (54.5)	19 (70.4)	49 (64.5)	40 (64.5)
	X^2 (df), P value	0.04 (1), 0.836		0.87 (1), 0.351		<0.001** (1), 0.996	
Paternal tobacco **Study Group N = 162 Control Group N=175**	Yes	18 (24.0)	27 (31.4)	5 (54.5)	7 (25.9)	20 (26.3)	17 (27.4)
	No	57 (76.0)	59 (68.6)	6 (54.5)	20 (74.1)	56 (73.7)	45 (72.6)
	X^2 (df), P value	1.08 (1), 0.297		1.38 (1), 0.240		0.02 (1), 0.884	
Paternal waterpipe smoking **Study Group N = 162 Control Group N=175**	Yes	10 (13.3)	3 (3.5)	3 (27.3)	1 (3.7)	9 (11.8)	5 (8.1)
	No	65 (86.7)	83 (96.5)	8 (72.7)	26 (96.3)	67 (88.2)	57 (91.9)
	X^2 (df), P value	5.23 (1), 0.022** 4.26 (1.13-16.1)		4.6 (1), 0.06		0.54 (1), 0.465	
Paternal Jorak **Study Group N = 162 Control Group N=175**	Yes	6 (8.0)	0 (0)	3 (27.3)	0 (0)	4 (5.3)	1 (1.6)
	No	69 (92.0)	86 (100)	8 (72.7)	27 (100)	72 (94.7)	61 (98.4)
	X^2 (df), P value	7.15 (1), 0.008**		8 (1), 0.005**		1.3 (1)0.254	
Paternal Moasel **Study Group N = 162 Control Group N=175**	Yes	5 (6.7)	3 (3.5)	1 (9.1)	1 (3.7)	6 (7.9)	4 (6.5)
	No	70 (93.3)	83 (96.5)	10 (90.9)	26 (96.3)	70 (92.1)	58 (93.5)
	X^2 (df), P value	0.86 (1), 0.335		0.46 (1), 0.5		0.11 (1), 0.745	
Maternal passive	Yes	16 (21.3)	18 (20.9)	5 (45.5)	2 (7.4)	12 (15.8)	12 (19.4)

smoking **Study Group N = 162** **Control Group N=165**	No	59 (78.7)	58 (79.1)	6 (54.5)	25 (92.6)	64 (84.2)	50 (80.6)
	X^2 (df), P value OR (95% CI)	0.004 (1), 0.950		7.53 (1), 0.006** 10.42 (1.61-67.34)		0.3 (1), 0.583	
Maternal stress							
Family problems **Study Group N = 162** **Control Group N=175**	Yes	26 (34.7)	25 (29.1)	7 (63.6)	4 (14.8)	30 (39.5)	14 (22.6)
	No	49 (65.3)	61 (70.9)	4 (36.4)	23 (85.7)	46 (60.5)	48 (77.4)
	X^2 (df), P value OR (95% CI)	0.58 (1), 0.446		9.06 (1), 0.003** 10.06 (1.98-51.04)		4.49 (1), 0.034** 2.24 (1.05-4.74)	
Mother complains of being under stress **Study Group N = 162** **Control Group N=175**	Yes	32 (42.7)	31 (36.0)	7 (63.6)	6 (22.2)	34 (44.7)	17 (27.4)
	No	43 (57.3)	55 (64.0)	4 (36.4)	21 (77.8)	42 (55.3)	45 (72.6)
	X^2 (df), P value OR (95% CI)	0.74 (1), 0.391		5.96 (1), 0.015** 6.13 (1.33-28.21)		4.4 (1), 0.036** 2.14 (1.04-4.39)	
Depression pre-gestation **Study Group N = 163** **Control Group N=173**	Yes	0 (0)	1 (1.2)	0 (0)	0 (0)	3 (3.9)	1 (1.7)
	No	75 (100)	85 (98.8)	11 (100)	27 (100)	74(96.1)	59 (98.3)
	X^2 (df), P value	0.88 (1), 0.349		a		0.59 (1), 0.442	
Depression 1st trimester **Study Group N = 163** **Control Group N=173**	Yes	2 (2.7)	3 (3.5)	2 (18.2)	0 (0)	3 (3.9)	0 (0)
	No	73 (97.3)	83 (96.5)	9 (81.8)	27 (100)	74(96.1)	60 (100)
	X^2 (df), P value	0.09 (1), 0.764		5.18 (1), 0.023**		2.39 (1), 0.122	
Severe morning sickness **Study Group N = 162** **Control Group N=173**	Yes	10 (13.3)	7 (8.1)	0 (0)	2 (7.4)	10 (13.2)	3 (5.0)
	No	65 (86.7)	79 (91.9)	11 (100)	25 (92.6)	66 (86.8)	57 (95.0)
	X^2 (df), P value	1.14 (1), 0.285		0.86 (1), 0.354		2.58 (1), 0.108	
Threatened abortion **Study Group N = 162** **Control Group N=173**	Yes	2 (2.7)	0 (0)	1 (9.1)	2 (7.4)	7 (9.2)	5 (8.3)
	No	73(97.3)	86 (100)	10 (90.9)	25 (92.6)	69 (90.8)	55 (91.7)
	X^2 (df), P value	2.32 (1), 0.128		0.03 (1), 0.861		0.03 (1), 0.858	
Abdominal pain pre-gestation **Study Group N = 162** **Control Group N=173**	Yes	2 (2.7)	4 (4.7)	0 (0)	0 (0)	4 (5.3)	1 (1.7)
	No	73 (97.3)	82 (95.3)	11 (100)	27 (100)	72 (94.7)	59 (98.3)
	X^2 (df), P value	0.44 (1), 0.507		a		1.22 (1), 0.17	
Abdominal pain 1st trimester	Yes	6 (8.0)	7 (6.1)	4 (36.4)	1 (3.7)	8 (10.5)	3 (5.0)
	No	69 (92.0)	79 (91.9)	7 (63.6)	26 (96.3)	68 (89.5)	57 (95.0)

Study Group N = 162 Control Group N=173	X^2 (df), P value OR (95% CI)	0.001 (1), 0.974		7.3 (1), 0.007** 14.86 (1.42-154.99)		1.38 (1), 0.241	
Maternal domestic environmental exposure							
Exposure to chemicals pre-gestation	Yes	25 (33.3)	24 (27.9)	6 (54.5)	4 (14.8)	20 (26.3)	19 (31.7)
	No	50 (66.7)	62 (72.1)	5 (45.5)	23 (85.7)	56 (73.7)	41 (68.3)
Study Group N = 162 Control Group N=173	X^2 (df), P value OR (95% CI)	0.56 (1), 0.455		6.36 (1), 0.012** 6.9 (1.4-33.92)		0.47 (1), 0.493	
Exposure to chemicals 1st trimester	Yes	25 (33.3)	26 (30.2)	6 (54.5)	4 (14.8)	18 (23.7)	19 (31.7)
	No	50 (66.7)	60 (69.8)	5 (45.5)	23 (85.7)	56 (76.3)	41 (68.3)
Study Group N = 160 Control Group N=173	X^2 (df), P value OR (95% CI)	0.18 (1), 0.673		6.36 (1), 0.012** 6.9 (1.4-33.92)		0.94 (1), 1.07 (1), 0.299	
Exposure to solvent pre-gestation	Yes	12 (16.0)	12 (14.0)	2 (18.2)	2 (7.4)	7 (9.2)	9 (14.5)
	No	63 (84.0)	74 (86.0)	9 (81.8)	25 (92.6)	69 (90.8)	53 (85.5)
Study Group N = 162 Control Group N=175	X^2 (df), P value	0.13 (1), 0.673		0.96 (1), 0.34		0.299	
Exposure to solvent 1st trimester	Yes	11 (14.7)	10 (11.6)	0 (0)	2 (7.4)	5 (6.6)	8 (12.9)
	No	64 (85.3)	76 (88.4)	11 (100)	25 (92.6)	71 (93.4)	54 (87.1)
Study Group N = 162 Control Group N=175	X^2 (df), P value	0.33 (1), 0.568		0.86 (1), 0.354		1.6 (1), 0.206	
Exposure to pesticides pre-gestation	Yes	11 (14.7)	14 (16.3)	1 (9.1)	5 (18.5)	12 (15.8)	13 (21.0)
	No	64 (85.3)	72 (83.7)	10 (90.9)	22 (81.5)	64 (84.2)	49 (79.0)
Study Group N = 162 Control Group N=175	X^2 (df), P value	0.08 (1), 0.778		0.52 (1), 0.470		0.62 (1), 0.432	
Exposure to pesticides 1st trimester	Yes	12 (16.0)	15 (17.4)	3 (27.3)	3 (11.1)	13 (17.1)	12 (19.4)
	No	63 (84.0)	71 (82.6)	8 (72.7)	24 (88.9)	63 (82.9)	50 (80.6)
Study Group N = 162 Control Group N=175	X^2 (df), P value	0.06 (1), 0.807		1.54 (1), 0.215		0.12 (1), 0.733	
Exposure to incense pre-gestation	Yes	31 (41.3)	45 (52.3)	4 (36.4)	9 (33.3)	23 (30.3)	30 (48.4)
	No	44 (58.7)	41 (47.7)	7 (63.6)	18 (66.7)	53 (69.7)	32 (51.6)
Study Group N = 162 Control Group N=175	X^2 (df), P value OR (95% CI)	1.94 (1), 0.163		0.03 (1), 0.858		4.74 (1), 0.029** 0.46 (0.23-0.93)	
Exposure to incense in the 1st trimester	Yes	30 (40.0)	46 (53.5)	4 (36.4)	8 (29.6)	24 (31.6)	31 (50.0)
	No	45 (60.0)	40 (46.5)	7 (63.6)	19 (70.4)	52 (68.4)	31 (50.0)

Study Group N = 162 Control Group N=175	X^2 (df), P value OR (95% CI)	2.9 (1), 0.087		0.16 (1), 0.685		4.8 (1), 0.028** 0.46 (0.23-0.92)	
Type of maternal drinking water *X2=* **Study Group N = 160 Control Group N=172**	Tap	23 (31.1)	17 (20)	1 (9.1)	2 (7.4)	16 (21.3)	15 (25)
	Bottled	46 (62.2)	57 (67.1)	10 (90.9)	20 (74.1)	51 (68)	40 (66.7)
		0.17		1		0.67	
	Well	5 (6.8)	1 (1.2)	0	5 (18.5)	5 (6.7)	1 (1.7)
		0.252		0.3		0.18	
	Zamzam	0	10 (11.8)	0	0	3 (4)	4 (6.7)
	P-value	0.024**		0.309		0.68	
Type of maternal drinking water	X^2 (df), P value	14.05 (3), 0.003		2.35 (3), 0.309		2.54 (3), 0.469	
Consanguinity **Study Group N = 157 Control Group N=164**	Yes	40 (57.1)	39 (49.4)	6 (50)	17 (68)	42 (56)	34 (56.7)
	No	30 (42.9)	40 (50.6)	6 (50)	8 (32)	33 (44)	26 (43.3)
	X^2 (df), P value	0.9 (1), 0.343		1.12 (1), 0.291		0.006 (1), 0.938	
Family history of birth defects **Study Group N = 162 Control Group N=172**	Yes	30 (40.0)	24 (28.9)	5 (54.5)	9 (33.3)	34 (44.7)	14 (22.6)
	No	45 (60.0)	59 (71.1)	6 (54.5)	18 (66.7)	42 (55.3)	48 (77.4)
	X^2 (df), P value OR (95% CI)	3.11 (1), 0.211		0.49 (1), 0.482		6.61 (1), 0.037** 2.78 (1.31—5.86)	

*The common homozygous allele genotype

**The Chi-square statistic is significant at the 0.05 level

[a] Not possible to analyse because the groups contain zero values

If one of the cells contains zero value, the OR and 95% CI are not calculated

A41: Relationship between maternal *IRF6* rs2235375 genotypes and different environmental factors, including: maternal illness, medication, stress, supplements, paternal smoking and maternal domestic exposure using case-only study design.

Maternal *IRF6* rs2235375		**CC*** (%)	GG (%)	CG (%)
Maternal medication use and illness				
Antibiotic pre-gestation X^2=0.1, df=2, P= 0.949 **N=163**	Yes	5 (14.7)	9 (13.8)	8 (12.5)
	No	29 (85.3)	56 (86.2)	56 (87.5)
	P value OR (95% CI)		0.907 0.93 (0.29-3.04)	0.76 0.83 (0.25-2.76)
Antibiotic at 1st trimester X^2=2.01, df=2, P= 0.365 **N=163**	Yes	4 (11.8)	8 (12.3)	13 (20.3)
	No	30 (88.2)	57 (87.7)	51 (79.7)
	P value OR (95% CI)		0.937 1.05 (0.29-3.78)	0.293 1.9 (0.57-6.4)
Antipyretic medication pre-gestation X^2=2.86, df=2, P= 0.239 **N=164**	Yes	4 (11.8)	5 (7.7)	2 (3.1)
	No	30 (88.2)	60 (92.3)	63 (96.9)
	P value OR (95% CI)		0.506 0.63 (0.16-2.5)	0.108 0.24 (0.04-1.37)
Antipyretic medication 1st trimester X^2=3.21, df=2, P= 0.201 **N=162**	Yes	6 (18.2)	5 (7.8)	5 (7.7)
	No	27 (81.8)	59 (92.2)	60 (92.3)
	P value OR (95% CI)		0.137 0.38 (0.11-1.35)	0.13 0.38 (0.11-1.33
Anti-emetic medication pre-gestation X^2=1.52, df=2, P= 0.468 **N=163**	Yes	0	1 (1.5)	0
	No	34 (100)	64 (98.5)	64 (100)
	P value OR (95% CI)		a	a
Anti-emetic medication 1st trimester X^2=0.29, df=2, P= 0.863 **N=163**	Yes	6 (17.6)	9 (13.8)	9 (14.1)
	No	28 (82.4)	56 (86.2)	55 (85.9)
	P value OR (95% CI)		0.617 0.75 (0.24-2.38)	0.64 0.75 (0.25-2.36)
Contraceptives pre-gestation X^2=2.7, df=2, P= 0.259 **N=161**	Yes	2 (6.2)	8 (12.3)	3 (4.7)
	No	30 (93.8)	57 (87.7)	61 (95.3)
	P value OR (95% CI)		0.365 2.11 (0.42-10.55)	0.746 0.74 (0.12-4.65)
Contraceptives 1st trimester X^2=2.9, df=2, P= 0.866 **N=130**	Yes	1 (2.9)	1 (3.5)	1 (1.6)
	No	33 (97.1)	31 (96.9)	63 (98.4)
	P value OR (95% CI)		0.965 1.06 (0.06-17.7)	0.651 0.52 (0.03-8.65)
Illness pre-gestation	Yes	10 (29.4)	17 (26.2)	18 (28.1)

X^2=0.13, df=2, P= 0.936	No	24 (70.6)	48 (73.8)	46 (71.9)
	P value OR (95% CI)		0.73 0.85 (0.34-2.14)	0.893 0.94 (0.38-2.35)
Illness 1st trimester X^2=2.81, df=2, P= 0.245 **N=163**	Yes	16 (47.1)	20 (30.8)	26 (40.6)
	No	18 (52.9)	45 (69.2)	38 (59.4)
	P value OR (95% CI)		0.112 0.5 (0.23-1.17)	0.541 0.77 (0.33-1.78)
Common cold/flu pre-gestation X^2=1.62, df=2, P= 0.445 **N=163**	Yes	7 (20.6)	16 (24.6)	10 (15.6)
	No	27 (79.4)	49 (75.4)	54 (84.4)
	P value OR (95% CI)		0.653 1.26 (0.56-3.44)	0.538 0.7 (0.24-2.08)
Common cold/flu 1st trimester X^2=0.27, df=2, P= 0.872 **N=163**	Yes	8 (23.5)	13 (20)	15 (23.4)
	No	26 (76.5)	52 (80)	49 (76.6)
	P value OR (95% CI)		0.684 0.81 (0.3-2.2)	0.684 0.99 (0.37-2.65)
Fever pre-gestation X^2=4.03, df=2, P= 0.13 **N=163**	Yes	4 (11.8)	10 (15.4)	3 (4.7)
	No	30 (88.2)	55 (84.6)	61 (95.3)
	P value OR (95% CI)		0.625 1.36 (0.39-4.7)	0.21 0.37 (0.08-1.75)
Fever 1st trimester X^2=1.07, df=2, P= 0.585 **N=163**	Yes	6 (17.6)	7 (10.8)	10 (15.6)
	No	28 (82.4)	58(89.2)	54 (84.4)
	P value OR (95% CI)		0.34 0.56 (0.17-1.83)	0.797 0.86 (0.28-2.62)
Urinary tract infection pre-gestation X^2=0.33, df=2, P= 0.85 **N=162**	Yes	1 (2.9)	2 (3.1)	3 (4.8)
	No	33 (97.1)	63 (96.9)	60 (95.2)
	P value OR (95% CI)		0.97 1.05 (0.09-11.99)	0.67 1.65 (0.17-16.5)
Urinary tract infection 1st trimester X^2=1.67, df=2, P= 0.433 **N=162**	Yes	0	2 (3.1)	3 (4.8)
	No	34 (100)	63 (96.9)	60 (95.2)
	P value OR (95% CI)		0.523 a	0.365 a
High blood pressure pre-gestation X^2=4.61, df=2, P= 0.1 **N=163**	Yes	0	3 (4.6)	0
	No	34 (100)	62 (100)	64 (100)
	P value OR (95% CI)		0.376 a	a
High blood pressure 1st trimester X^2=6.18, df=2, P= 0.045** **N=163**	Yes	0	4 (6.2)	0
	No	34 (100)	61 (93.8)	64 (100)
	P value OR (95% CI)		0.282 a	a
Diabetes pre-gestation **N=163**	Yes	0	0	0
	No	34 (100)	65 (100)	64 (100)
	P value		a	a

	OR (95% CI)			
Diabetes 1st trimester X^2=3.43, df=2, P= 0.18 **N=163**	Yes	2 (5.9)	0	2 (3.1)
	No	32 (94.1)	65 (100)	62 (96.9)
	P value OR (95% CI)		a	0.518 0.52 (0.06-3.85)
Asthma pre-gestation X^2=1.2, df=2, P= 0.548 **N=162**	Yes	0	2 (3.1)	1 (1.6)
	No	34 (100)	63 (96.9)	62 (100)
	P value OR (95% CI)		0.523a	a
Asthma 1st trimester X^2=4.56, df=2, P= 0.102 **N=162**	Yes	0	3 (4.6)	0
	No	34 (100)	62 (95.4)	63 (100)
	P value OR (95% CI)		0.376a	a
Convulsions pre-gestation A **N=163**	Yes	0	0	0
	No	34 (100)	65 (100)	64 (100)
	P value OR (95% CI)		a	a
Convulsions 1st trimester A **N=163**	Yes	0	0	0
	No	34 (100)	65 (100)	64 (100)
	P value OR (95% CI)		a	a
Vaginal bleeding X^2=3.32, df=2, P= 0.19 **N=162**	Yes	2 (5.9)	2 (3.1)	7 (11.1)
	No	32 (94.1)	63 (96.9)	56 (88.9)
	P value OR (95% CI)		0.508 0.51 (0.069-3.77)	0.405 2 (0.39-10.21)
Maternal exposure to X-ray 1st trimester X^2=1.63, df=2, P= 0.443 **N=164**	Yes	0	3 (4.9)	3 (4.9)
	No	34 (100)	62 (95.4)	62 (95.4)
	P value OR (95% CI)		0.376	0.376
Maternal supplement use				
Folic acid pre-gestation X^2=4.43, df=2, P= 0.109 **N=164**	Yes	6 (17.6)	3 (4.6)	7 (10.8)
	No	28 (82.4)	62 (95.4)	58 (89.2)
	P value OR (95% CI)		0.045** 0.23 (0.05-0.97)	0.34 0.56 (0.17-1.83)
Folic acid 1st trimester X^2=0.13, df=2, P= 0.938 **N=164**	Yes	20 (58.8)	37 (56.9)	39 (60)
	No	14 (41.2)	28 (43.1)	26 (40)
	P value OR (95% CI)		0.856 .93 (0.4-2.15)	0.91 1.05 (0.45-2.44)
Multivitamins pre-gestation X^2=2.41, df=2, P= 0.3	Yes	4 (11.2)	3 (4.6)	3 (4.6)
	No	30 (88.2)	62 (95.4)	62 (95.4)

N=164	P value OR (95% CI)		0.203 0.36 (0.08-1.73)	0.203 0.36 (0.08-1.73)
Multivitamins 1st trimester X^2=2.27, df=2, P= 0.32 **N=181**	Yes	10 (29.4)	12 (18.5)	11 (17.2)
	No	42 (70.6)	53 (81.5)	53 (82.8)
	P value OR (95% CI)		0.916 0.95 (0.37-2.41)	0.776 0.87 (0.34-2.25)
Iron pre-gestation X^2=1.22, df=2, P= 0.543 **N=164**	Yes	4 (11.8)	4 (6.2)	8 (2.9)
	No	30 (88.2)	61 (93.8)	57 (97.1)
	P value OR (95% CI)		0.339 0.49 (0.11-2.1)	0.937 1.05 (0.29-3.78)
Iron 1st trimester X^2=2.39, df=2, P= 0.302 **N=164**	Yes	13 (38.2)	23 (51.4)	18 (25.7)
	No	21 (61.8)	42 (64.6)	47 (74.3)
	P value OR (95% CI)		0.78 0.88 (0.37-2.09)	0.285 0.62 (0.26-1.49)
Calcium 1st trimester X^2=0.5, df=2, P= 0.777 **N=196**	Yes	1 (2.9)	4 (6.2)	3 (4.7)
	No	66 (97.1)	61 (93.8)	61 (95.3)
	P value OR (95% CI)		0.196 .33 (0.47-39.8)	0.314 3.25 (0.33-32.05)
Smoking				
Maternal smoking X^2=5.38, df=2, P= 0.068 **N=162**	Yes	1 (2.9)	5 (7.7)	0
	No	33 (97.1)	60 (92.3)	63 (100)
	P value OR (95% CI)		0.365 2.7 (0.31-24.5)	0.291 a
Paternal smoking X^2=1.49, df=2, P= 0.476 **N=162**	Yes	9 (26.5)	25 (38.5)	23 (36.5)
	No	25 (73.5)	40 (61.5)	40 (63.5)
	P value OR (95% CI)		0.235 1.7 (0.7-4.32)	0.318 1.6 (0.64-4)
Paternal tobacco X^2=1.75, df=2, P= 0.416 **N=171**	Yes	6 (17.6)	19 (29.2)	18 (28.6)
	No	28 (82.4)	46 (70.8)	54 (71.4)
	P value OR (95% CI)		0.212 1.93 (0.69-5.41)	0.4 1.56 (0.56-4.36)
Paternal waterpipe X^2=3.31, df=2, P= 0.191 **N=162**	Yes	7 (20.6)	10 (15.4)	5 (7.9)
	No	27 (79.4)	55 (84.6)	58 (92.1)
	P value OR (95% CI)		0.516 0.7 (0.24-2.04)	0.08 0.33 (0.1-1.14)
Paternal Jorak X^2=0.89, df=2, P= 0.64 **N=162**	Yes	4 (11.8)	5 (7.7)	4 (6.3)
	No	30 (88.2)	60 (92.3)	59 (93.7)
	P value OR (95% CI)		0.506 0.63 (0.16-2.5)	0.362 0.51 (0.12-2.18)

Paternal Moasel X^2=2.9, df=2, P= 0.234 **N=162**	Yes	4 (11.8)	6 (9.2)	2 (3.2)
	No	30 (88.2)	59 (90.8)	61 (96.8)
	P value OR (95% CI)		0.692 0.76 (0.2-2.91)	0.117 0.25 (0.04-1.42)
Maternal passive smoking X^2=1.29, df=2, P= 0.525 **N=162**	Yes	8 (23.5)	15 (23.1)	10 (15.9)
	No	26 (76.5)	50 (76.9)	53 (84.1)
	P value OR (95% CI)		0.96 0.98 (0.37-2.6)	0.357 0.61 (0.22-1.74)
Maternal stress				
Family problems X^2=2.48, df=2, P= 0.29 **N=162**	Yes	17 (50)	22 (33.8)	24 (38.1)
	No	17 (50)	43 (66.2)	39 (61.9)
	P value OR (95% CI)		0.121 0.51 (0.22-1.19)	0.259 0.62 (0.26-1.43)
Mother complains of being under stress X^2=0.69, df=2, P= 0.71 **N=147**	Yes	17 (50)	12 (41.5)	29 (46)
	No	17 (50)	38 (58.5)	34 (54)
	P value OR (95% CI)		0.016** 0.3 (0.1-0.8)	0.709 0.85 (0.37-1.97)
Depression pre-gestation X^2=4.73, df=2, P= 0.094 **N=163**	Yes	0	0	3 (4.7)
	No	34 (100)	65 (100)	61 (95.3)
	P value OR (95% CI)		a	0.37 a
Depression 1st trimester X^2=0.47, df=2, P= 0.792 **N=163**	Yes	2 (5.9)	2 (3.1)	3 (4.7)
	No	32 (94.1)	63 (96.9)	61 (95.3)
	P value OR (95% CI)		0.51 0.51 (0.07-3.77)	0.799 0.79 (0.13-5)
Severe morning sickness X^2=0.02, df=2, P= 0.991 **N=162**	Yes	4 (11.8)	8 (12.3)	8 (12.7)
	No	30 (88.2)	57 (87.7)	55 (87.3)
	P value OR (95% CI)		0.937 .05 (0.29-3.78)	0.894 1.09 (0.3-3.9)
Threatened abortion X^2=2.3, df=2, P= 0.316 **N=162**	Yes	2 (5.9)	2 (3.1)	6 (9.5)
	No	32 (94.1)	63 (96.9)	57 (90.5)
	P value OR (95% CI)		0.508 0.51 (0.069-3.77)	0.538 1.68 (0.32-8.84)
Abdominal pain pre-gestation X^2=057, df=2, P= 0.751 **N=162**	Yes	2 (5.9)	2 (3.1)	2 (3.2)
	No	32 (94.1)	63 (96.9)	61 (96.8)
	P value OR (95% CI)		0.508 0.51 (0.069-3.77)	0.529 0.52 (0.07-3.9)
Abdominal pain 1st trimester X^2=1.86, df=2, P= 0.394	Yes	6 (17.6)	6 (9.2)	6 (9.5)
	No	28 (82.4)	59 (90.8)	57 (90.5)

N=162	P value OR (95% CI)		0.23 0.47 (0.14-1.6)	0.253 0.49 (0.15-1.66)
Maternal domestic environmental exposure				
Exposure to chemicals pre-gestation X^2=0.99, df=2, P= 0.61 **N=162**	Yes	12 (35.3)	22 (33.8)	17 (27)
	No	22 (64.7)	43 (66.2)	46 (73)
	P value OR (95% CI)		0.886 0.94 (0.39-2.24)	0.395 0.68 (0.28-1.66)
Exposure to chemicals 1st trimester X^2=0.73, df=2, P= 0.695	Yes	10 (29.4)	22 (33.8)	17 (27)
	No	24 (70.6)	43 (66.2)	46 (73)
	P value OR (95% CI)		0.654 1.23 (0.5-3.02)	0.799 0.89 (0.35-2.23)
Exposure to solvents pre-gestation X^2=0.57, df=2, P= 0.751 **N=162**	Yes	4 (11.8)	10 (15.4)	7 (11.1)
	No	30 (88.2	55 (84.6)	56 (88.9)
	P value OR (95% CI)		0.625 1.36 (0.39-4.72)	0.923 0.94 (0.25-3.46)
Exposure to solvents 1st trimester X^2=0.77, df=2, P= 0.679 **N=162**	Yes	2 (5.9)	7 (10.8)	7 (11.1)
	No	32 (94.1)	58 (89.2)	56 (88.9)
	P value OR (95% CI)		0.429 0.93 (0.38-9.85)	0.405 2 (0.39-10.23)
Exposure to pesticides pre-gestation X^2=0.11, df=2, P= 0.949 **N=162**	Yes	5 (14.7)	9 (13.8)	10 (15.9)
	No	29 (85.3)	56 (86.2)	53 (84.1)
	P value OR (95% CI)		0.907 0.93 (0.29-3.04)	0.88 1.09 (0.34-3.5)
Exposure to pesticides 1st trimester X^2=0.22, df=2, P= 0.895 **N=131**	Yes	5 (14.7)	5 (14.7)	11 (17.5)
	No	29 (85.3)	29 (85.3)	52 (82.5)
	P value OR (95% CI)		1 1 (0.26-3.83)	0.728 1.23 (0.39-3.9)
Exposure to incense pre-gestation X^2=1.86, df=2, P= 0.395 **N=139**	Yes	9 (26.5)	25 (38.5)	13 (32.5)
	No	25 (73.5)	40 (61.5)	27 (67.5)
	P value OR (95% CI)		0.235 1.7 (0.7-4.32)	0.572 1.34 (0.49-3.67)
Exposure to incense 1st trimester X^2=1.86, df=2, P= 0.395 **N=139**	Yes	9 (26.5)	25 (38.5)	12 (30)
	No	25 (73.5)	40 (61.5)	28 (70)
	P value OR (95% CI)		0.235 1.7 (0.7-4.32)	0.737 1.19 (0.43-3.3)
Maternal exposure to X-ray	Yes	0	3 (4.9)	3 (4.9)

1st trimester X^2=1.63, df=2, P= 0.443 **N=164**	No	34 (100)	62 (95.4)	62 (95.4)
	P value OR (95% CI)		0.376 a	0.376 a
Type of maternal drinking water X^2=4.93, df=2, P= 0.553 **N=160**	Tap (reference)	8 (23.5)	20 (31.2)	12 (19.4)
	Bottled	23 (67.6)	39 (60.9)	45 (72.6)
	P value OR (95% CI)		0.432 1.47 (0.56-2.38)	0.612 0.77 (0.27-2.14)
	Well	2 (5.9)	5 (7.8)	3 (4.8)
	P value OR (95% CI)		1 1 (0.16-6.26)	1 1(0.14-7.39)
	Zamzam	1 (2.9)	0	2 (3.2)
	P value OR (95% CI)		0.24 a	0.826 0.75 (0.06-9.72)
	OR (95% CI)	a	a	a
Consanguinity X^2=3.85, df=2, P= 0.146 **N=157**	Yes	15 (42.9)	38 (63.3)	36 (58.1)
	No	20 (57.1)	22 (36.7)	26 (41.9)
	P value OR (95% CI)		0.551 2.3 (0.98-5.39)	0.15 1.85 (0.8-4.27)
Family history X^2=2.61, df=2, P= 0.626 **N=160**	Yes	14 (41.2)	24 (36.9)	29 (46)
	No	19 (55.9)	40 (61.5)	34 (54)
	P value OR (95% CI)		0.638 0.81 (0.35-1.92)	0.736 1.16 (0.49-2.71)

*Homozygous common allele genotype.
**The Chi-square statistic is significant at the 0.05 level.

[a] Could not analyse because the groups contained zero values.
If one of the cells contained a zero value, the OR and 95% CI were not calculated.

A42: Relationship between maternal *IRF6* rs2235375 allele frequency and different environmental factors, including: maternal illness, medication, stress, supplements, paternal smoking and maternal domestic exposure using case-only study design.

Maternal IRF6 rs2235375		C* (%)	G (%)
Maternal medication use and illness			
Antibiotic pre-gestation **N=326**	Yes	18 (13.6)	26 (13.4)
	No	114 (86.4)	168 (86.6)
	X^2 (df), P value OR (95% CI)	0.00 (1) 0.952 0.98 (0.51-1.87)	
Antibiotic at 1st trimester **N=326**	Yes	21 (15.9)	29 (14.9)
	No	111(84.1)	165 (85.1)
	X^2 (df), P value OR (95% CI)	0.06 (1) 0.81 0.93 (0.5-1.7)	
Antipyretic medication pre-gestation **N=328**	Yes	10 (7.5)	12 (6.2)
	No	123 (92.5)	183 (93.8)
	X^2 (df), P value OR (95% CI)	0.24 (1) 0.628 0.81 (0.34-1.92)	
Antipyretic medication 1st trimester **N=324**	Yes	17 (13)	15 (7.8)
	No	114 (87)	178 (92.2)
	X^2 (df), P value OR (95% CI)	2.38 (1) 0.127 0.57 (0.27-1.18)	
Anti-emetic medication pre-gestation **N=326**	Yes	0	2 (1)
	No	132 (100)	192 (99)
	X^2 (df), P value OR (95% CI)	1.37 (1) 0.24 3.44 (0.16-72.27)	
Anti-emetic medication 1st trimester **N=326**	Yes	21 (15.9)	27 (13.9)
	No	111 (84.1)	167 (86.1)
	X^2 (df), P value OR (95% CI)	0.25 (1) 0.62 0.85 (0.46-1.59)	
Contraceptives pre-gestation	Yes	7 (5.5)	19 (9.8)
	No	121 (94.5)	175 (90.2)
	X^2 (df), P value OR (95% CI)	1.94 (1) 0.16 1.88 (0.77-4.6	
Contraceptives 1st trimester **N=260**	Yes	3 (2.3)	3 (2.3)
	No	129 (97.7)	125 (97.7)
	X^2 (df), P value OR (95% CI)	0.00 (1) 0.97 1.03 (0.2-5.21)	

Illness pre-gestation **N=326**	Yes	38 (28.8)	52 (26.8)
	No	94 (71.2)	142 (73.2)
	X^2 (df), P value OR (95% CI)	0.15 (1) 0.69 0.91 (0.55-1.48)	
Illness 1st trimester **N=326**	Yes	58 (43.9)	66 (34)
	No	74 (56.1)	128 (66)
	X^2 (df), P value OR (95% CI)	3.28 (1) 0.071 0.66 (0.42-1.04)	
Common cold/flu pre-gestation **N=326**	Yes	24 (18.2)	42 (21.7)
	No	108 (81.8)	152 (78.3)
	X^2 (df), P value OR (95% CI)	0.59 (1) 0.44 1.24 (0.71-2.17)	
Common cold/flu 1st trimester **N=326**	Yes	31 (23.5)	41 (21.1)
	No	101 (76.5)	153 (78.9)
	X^2 (df), P value OR (95% CI)	0.25 (1) 0.62 0.87 (0.51-1.48)	
Fever pre-gestation **N=326**	Yes	11 (8.3)	23 (11.9)
	No	121 (91.7)	171 (88.1)
	X^2 (df), P value OR (95% CI)	1.04 (1) 0.309 1.48 (0.7-3.15)	
Fever 1st trimester **N=326**	Yes	22 (16.7)	24 (12.4)
	No	110 (83.3)	170 (87.6)
	X^2 (df), P value OR (95% CI)	1.20 (1) 0.27 0.71 (0.38-1.32)	
Urinary tract infection pre-gestation **N=324**	Yes	5 (3.8)	7 (3.6)
	No	126 (96.2)	186 (96.4)
	X^2 (df), P value OR (95% CI)	0.01 (1) 0.93 0.95 (0.29-3.05)	
Urinary tract infection 1st trimester **N=324**	Yes	3 (2.3)	7 (3.6)
	No	128 (97.7)	186 (96.4)
	X^2 (df), P value OR (95% CI)	0.47 (1) 0.49 1.61 (0.41-6.33)	
High blood pressure pre-gestation **N=326**	Yes	0	6 (3.1)
	No	132 (100)	188 (96.9)
	X^2 (df), P value	4.16 (1) 0.04	
High blood pressure 1st trimester **N=334**	Yes	0	8 (4)
	No	132 (100)	194 (96)
	X^2 (df), P value	5.36 (1) 0.02	
Diabetes pre-gestation **N=326**	Yes	0	0
	No	132 (100)	194 (100)

	X^2 (df), P value	a	
Diabetes 1st trimester **N=326**	Yes	6 (4.6)	2 (1)
	No	126 (95.4)	192 (99)
	X^2 (df), P value OR (95% CI)	4.05 (1) 0.04 0.22 (0.04-1.1)	
Asthma pre-gestation **N=324**	Yes	1 (0.8)	5 (2.6)
	No	130 (99.2)	188 (97.4)
	X^2 (df), P value OR (95% CI)	1.43 (1) 0.23 3.46 (0.4-29.94)	
Asthma 1st trimester **N=324**	Yes	0	6 (3.1)
	No	131 (100)	187 (96.9)
	X^2 (df), P value	4.15 (1) 0.04	
Convulsions pre-gestation **N=326**	Yes	0	0
	No	132 (100)	194 (100)
	X^2 (df), P value	a	
Convulsions 1st trimester **N=326**	Yes	0	0
	No	132 (100)	194 (100)
	X^2 (df), P value	a	
Vaginal bleeding **N=324**	Yes	11 (8.4)	11 (5.7)
	No	120 (91.6)	182 (94.3)
	X^2 (df), P value OR (95% CI)	0.90 (1) 0.346 0.66 (0.28-1.57)	
Maternal supplement use			
Folic acid pre-gestation **N=324**	Yes	11 (8.4)	11 (5.7)
	No	120 (91.6)	182 (94.3)
	X^2 (df), P value OR (95% CI)	0.89 (1) 0.34 0.83 (0.54-1.27)	
Folic acid 1st trimester **N=328**	Yes	79 (59.4)	113 (58)
	No	54 (40.6)	82 (42)
	X^2 (df), P value OR (95% CI)	0.07 (1) 0.793 0.98 (0.81-1.17)	
Multivitamins pre-gestation **N=328**	Yes	11 (8.3)	9 (4.6)
	No	122 (91.7)	186 (95.4)
	X^2 (df), P value OR (95% CI)	1.85 (1), 0.17 0.75 (0.46-1.22)	
Multivitamins 1st trimester **N=362**	Yes	31 (18.5)	35 (18)
	No	137 (81.5)	159 (82)
	X^2 (df), P value OR (95% CI)	0.01 (1), 0.92 0.97 (0.57-1.66)	
Iron pre-gestation **N=328**	Yes	16 (12)	16 (8.2)
	No	117 (88)	179 (91.8)
	X^2 (df), P value	1.31 (1), 0.254	

	OR (95% CI)	0.65 (0.31-1.36)	
Iron 1st trimester **N=328**	Yes	44 (33.1)	64 (32.8)
	No	89 (66.9)	131 (67.2)
	X^2 (df), P value OR (95% CI)	0.00 (1) 0.96 0.99 (0.62-1.58)	
Calcium 1st trimester **N=392**	Yes	5 (2.5)	11 (5.7)
	No	193 (97.5)	183 (94.3)
	X^2 (df), P value OR (95% CI)	2.48 (1) 0.125 2.32 (0.79-6.81)	
Smoking			
Maternal smoking **N=324**	Yes	2 (1.5)	10 (5.2)
	No	129 (98.5)	183 (94.8)
	X^2 (df), P value OR (95% CI)	2.92 (1) 0.09 3.52 (0.76-16.36)	
Paternal smoking **N=324**	Yes	41 (31.3)	73 (37.8)
	No	90 (68.7)	120 (62.2)
	X^2 (df), P value OR (95% CI)	1.46 (1) 0.23 1.34 (0.83-2.14)	
Paternal tobacco **N=342**	Yes	30 (21.4)	56 (37.7)
	No	110 (78.6)	146 (72.3)
	X^2 (df), P value OR (95% CI)	1.74 (1) 0.19 1.41 (0.85-2.34)	
Paternal waterpipe **N=324**	Yes	19 (14.5)	25 (13)
	No	112 (85.5)	168 (87)
	X^2 (df), P value OR (95% CI)	0.16 (1) 0.689 0.88 (0.46-1.67)	
Maternal passive smoking **N=366**	Yes	26 (17.7)	40 (18.3)
	No	121 (82.3)	179 (81.7)
	X^2 (df), P value OR (95% CI)	0.02 (1) 0.89 1.04 (0.6-1.79)	
Maternal stress			
Family problems **N=324**	Yes	58(44.3)	68 (35.2)
	No	73 (55.7)	125 (64.8)
	X^2 (df), P value OR (95% CI)	2.68 (1) 0.10 0.68 (0.423-1.08)	
Mother complains of being under stress **N=294**	Yes	63 (48.1)	53 (32.5)
	No	68 (51.9)	110 (67.5)
	X^2 (df), P value OR (95% CI)	7.38 (1) 0.01** 0.52 (0.32-0.84)	
Depression pre-gestation **N=326**	Yes	3 (2.3)	3 (1.6)
	No	129 (97.7)	191 (98.4)

	X^2 (df), P value OR (95% CI)	0.23 (1) 0.634 0.675 (0.13-3.4)	
Depression 1st trimester **N=326**	Yes	7 (5.3)	7 (3.6)
	No	125 (94.7)	187 (96.4)
	X^2 (df), P value OR (95% CI)	0.55 (1) 0.46 0.67 (0.23-1.95)	
Severe morning sickness **N=324**	Yes	16 (21.2)	24 (12.4)
	No	115 (87.8)	169 (87.6)
	X^2 (df), P value OR (95% CI)	0.00 (1) 0.95 1.02 (0.52-2.01)	
Threatened abortion **N=324**	Yes	10 (7.6)	10 (5.2)
	No	121 (92.4)	183 (94.8)
	X^2 (df), P value OR (95% CI)	0.81 (1) 0.37 0.66 (0.27-1.64)	
Abdominal pain pre-gestation **N=324**	Yes	6 (4.6)	6 (3.1)
	No	125 (95.4)	187 (96.9)
	X^2 (df), P value OR (95% CI)	0.47 (1) 0.49 0.48 (0.15-01.52)	
Abdominal pain 1st trimester **N=344**	Yes	18 (11.9)	18 (9.3)
	No	133 (88.1)	175 (90.7)
	X^2 (df), P value OR (95% CI)	0.61 (1) 0.44 0.76 (0.38-1.52)	
Maternal domestic environmental exposure			
Exposure to chemicals pre-gestation **N=324**	Yes	41 (31.3)	61 (31.6)
	No	90 (68.7)	132 (68.4)
	X^2 (df), P value OR (95% CI)	0.00 (1) 0.953 1.01 (0.63-1.634)	
Exposure to chemicals 1st trimester **N=320**	Yes	37 (29.1)	61 (31.6)
	No	90 (70.9)	132 (68.4)
	X^2 (df), P value OR (95% CI)	0.22 (1) 0.639 1.12 (0.69-1.69)	
Exposure to solvents pre-gestation **N=324**	Yes	15 (11.5)	27 (14)
	No	116 (88.5)	166 (86)
	X^2 (df), P value OR (95% CI)	0.45 (1) 0.505 1.26 (0.64-2.47)	
Exposure to solvents 1st trimester **N=324**	Yes	11 (8.4)	21 (10.9)
	No	120 (91.6)	172 (89.1)
	X^2 (df), P value OR (95% CI)	0.54 (1) 0.463 1.33 (0.62-2.86)	
Exposure to pesticides pre-gestation	Yes	20 (15.3)	28 (14.5)
	No	111 (84.7)	165 (85.5)

N=324	X^2 (df), P value OR (95% CI)	0.04 (1) 0.85 0.94 (0.51-1.75)	
Exposure to pesticides 1st trimester **N=262**	Yes	21 (16)	21 (16)
	No	110 (84)	110 (84)
	X^2 (df), P value OR (95% CI)	0 (1) 1 1 (0.52-1.94)	
Exposure to incense pre-gestation **N=278**	Yes	31 (28.7)	63 (37.1)
	No	77 (71.3)	107 (62.9)
	X^2 (df), P value OR (95% CI)	2.06 (1) 0.15 1.46 (0.87-2.46)	
Exposure to incense 1st trimester **N=278**	Yes	30 (27.8)	62 (36.5)
	No	78 (72.1)	108 (63.5)
	X^2 (df), P value OR (95% CI)	2.25 (1) 0.13 1.49 (0.88-2.52)	
Maternal exposure to X-ray 1st trimester **N=211**	Yes	3 (2.3)	9 (4.6)
	No	13(97.7)	186 (95.4)
	X^2 (df), P value OR (95% CI)	5.51 (1) 0.02** 0.21 (0.05-0.87)	
Type of maternal drinking water 3.32 (1) 0.35 **N=320**	Tap (reference)	28 (21.5)	52 (27.4)
	Bottled	91 (70)	123 (74.7)
	X^2 (df), P value OR (95% CI)	1.37 (1) 0.243 1.37 (0.8-2.34)	
	Well	7 (5.4)	13 (6.8)
	OR (95% CI)	1	1 (0.36-2.79)
	Zamzam	4 (3.1)	2 (1.1)
	P value OR (95% CI)	0.144	3.71 (0.64-21.56)
Consanguinity **N=314**	Yes	66 (50)	112 (61.5)
	No	66 (50)	70 (38.5)
	X^2 (df), P value OR (95% CI)	4.15 (1) 0.04** 1.6 (1.02-2.52)	
Family history **N=320**	Yes	57 (44.2)	77 (40.3)
	No	72 (55.8)	114 (59.7)
	X^2 (df), P value OR (95% CI)	0.47 (1) 0.491 0.85 (0.54-1.34)	

*Common allele.

**Significant level P≤ 0.05

[a] Not possible to analyse because the groups contain zero values

If one of the cells contains zero value, the OR and 95% CI are not calculated

A43: Comparison between cases and controls for maternal *IRF6* rs2235375 genotypes in relationship to different environmental factors including: maternal illness, medication, stress, supplements, paternal smoking and maternal domestic exposure.

Maternal *IRF6* rs2235375		**CC*** (%)		GG (%)		CG (%)	
Environmental factors		Study	Control	Study	Control	Study	Control
Maternal medication use and illness							
Antibiotic pre-gestation **Study Group N=163 Control Group N=179**	Yes	5 (14.7)	1 (2.5)	9 (13.8)	4 (5.2)	8 (12.5)	4 (6.5)
	No	29 (85.3)	39 (97.5)	56 (86.2)	73 (94.8)	56 (87.5)	58 (93.5)
	X^2 (df), P value	3.68 (1), 0.055		3.17 (1), 0.075		1.34 (1), 0.248	
Antibiotic at 1st trimester **Study Group N=163 Control Group N=179**	Yes	4 (11.8)	2 (5)	8 (12.3)	6 (7.8)	13 (20.3)	10 (16.1)
	No	30 (88.2)	38 (95)	57 (87.7)	71 (92.2)	51 (79.7)	52 (83.9)
	X^2 (df), P value	1.13 (1), 0.288		0.81 (1), 0.369		0.37 (1), 0.543	
Antipyretic medication pre-gestation **Study Group N=164 Control Group N=179**	Yes	4 (11.8)	5 (12.5)	5 (7.7)	8 (10.4)	2 (3.1)	5 (8.1)
	No	30 (88.2)	35 (87.5)	60 (92.3)	69 (89.6)	63 (96.9)	57 (91.9)
	X^2 (df), P value	0.009 (1), 0.923		0.31 (1), 0.579		1.52 (1), 0.218	
Antipyretic medication 1st trimester **Study Group N=162 Control Group N=176**	Yes	6 (18.2)	3 (7.7)	5 (7.8)	11 (14.5)	5 (7.7)	11 (18)
	No	27 (81.8)	36 (92.3)	59 (92.2)	65 (85.5)	60 (92.3)	50 (82)
	X^2 (df), P value	1.8 (1), 0.18		1.52 (1), 0.217		3.04 (1), 0.081	
Anti-emetic medication pre-gestation **Study Group N=162 Control Group N=179**	Yes	0	0	1 (1.5)	0	0	0
	No	34 (100)	40 (100)	64 (98.5)	77 (100)	64 (100)	62 (100)
	X^2 (df), P value	a		1.19 (1), 0.275		a	
Anti-emetic medication 1st trimester **Study Group N=163 Control Group N=179**	Yes	6 (17.6)	3 (7.5)	9 (13.8)	5 (6.6)	9 (14.1)	6 (9.7)
	No	28 (82.4)	37 (92.5)	56 (86.2)	72 (91)	55 (85.9)(1),	56 (90.3)
	X^2 (df), P value	1.77 (1), 0.183		2.14 (1), 0.143		0.58(1), 0.447	
Contraceptives pre-gestation **Study Group N=161 Control Group N=179**	Yes	2 (6.2)	4 (10)	8 (12.3)	4 (5.2)	3 (4.7)	6 (9.7)
	No	30 (93.8)	36 (90)	57 (87.7)	73 (94.8)	61 (95.3)	56 (90.3)
	X^2 (df), P value	0.32 (1), 0.567		2.31 (1), 0.129		1.18 (1), 0.227	

Contraceptives 1st trimester **Study Group N=130** **Control Group N=138**	Yes	1 (2.9)	2 (5)	1 (1.5)	2 (2.6)	1 (1.6)	1 (1.6)
	No	33 (97.1)	38 (95)	31 (96.9)	34 (97.1)	63 (98.4)	61 (98.4)
	X^2 (df), P value	0.19 (1), 0.654		0.2 (1), 0.662		0.001 (1), 0.982	
Illness pre-gestation **Study Group N=163** **Control Group N=178**	Yes	10 (29.4)	2 (5)	17 (26.2)	11 (14.3)	18 (28.1)	6 (9.8)
	No	24 (70.6)	38 (95)	48 (73.8)	66 (85.7)	46 (71.9)	55 (90.2)
	X^2 (df), P value OR (95% CI)	8.06 (1), 0.005** 7.92 (1.639.29)		3.14 (1), 0.077		6.73 (1), 0.009** 3.59 (1.31-9.78)	
Illness 1st trimester **Study Group N=163** **Control Group N=177**	Yes	16 (47.1)	8 (20)	20 (30.8)	21 (27.6)	26 (40.6)	18 (29.5)
	No	18 (52.9)	32 (80)	45 (69.2)	55 (72.4)	38 (59.4)	43 (70.5)
	X^2 (df), P value OR (95% CI)	6.14 (1), 0.013** 3.56(1.27-9.92)		0.17 (1), 0.683		1.69 (1), 0.193	
Common cold/flu pre-gestation **Study Group N=163** **Control Group N=177**	Yes	7 (20.6)	1 (2.5)	16 (24.6)	2 (2.6)	10 (15.6)	4 (6.6)
	No	27 (79.4)	39 (97.5)	49 (75.4)	74 (97.4)	54 (84.4)	57 (93.4)
	X^2 (df), P value OR (95% CI)	6.24 (1), 0.013** 10.11 (1.18-86.98)		15.2 (1), <0.001** 12.08 (2.66-54.89)		2.58 (1), 0.108	
Common cold/flu 1st trimester **Study Group N=163** **Control Group N=177**	Yes	8 (23.5)	3 (7.5)	13 (20)	9 (11.8)	15 (23.4)	12 (19.7)
	No	26 (76.5)	37 (92.5)	52 (80)	67 (88.2)	49 (76.6)	49 (80.3)
	X^2 (df), P value	3.73 (1), 0.053		1.77 (1), 0.183		0.26 (1), 0.609	
Fever pre-gestation **Study Group N=163** **Control Group N=177**	Yes	4 (11.8)	1 (2.5)	10 (15.4)	2 (2.6)	3 (4.7)	5 (8.2)
	No	30 (88.2)	39 (97.5)	55 (84.6)	74 (97.4)	61 (95.3)	56 (91.8)
	X^2 (df), P value OR (95% CI)	2.5 (1), 0.114		7.32 (1), 0.007** 6.73 (1.42-31.94)		0.64 (1), 0.423	
Fever 1st trimester **Study Group N=163** **Control Group N=177**	Yes	6 (17.6)	2 (5)	7 (10.8)	7 (9.2)	10 (15.6)	6 (9.8)
	No	28 (82.4)	38 (95)	58(89.2)	69 (90.8)	54 (84.4)	55 (90.2)
	X^2 (df), P value	3.05 (1), 0.081		0.1 (1), 0.758		0.94 (1), 0.333	
Urinary tract infection pre-gestation **Study Group N=162** **Control Group N=177**	Yes	1 (2.9)	1 (2.5)	2 (3.1)	1 (1.3)	3 (4.8)	2 (3.3)
	No	33 (97.1)	39 (97.5)	63 (96.9)	75 (98.7)	60 (95.2)	59 (96.7)
	X^2 (df), P value	0.01 (1), 0.907		0.52 (1), 0.483		0.18 (1), 0.676	
Urinary tract infection 1st trimester **Study Group N=162** **Control Group N=177**	Yes	0	1 (2.5)	2 (3.1)	3 (3.9)	3 (4.8)	3 (4.9)
	No	34 (100)	39 (97.5)	63 (96.9)	73 (96.1)	60 (95.2)	58 (95.1)
	X^2 (df), P value	0.86 (1), 0.57		0.08 (1), 0.781		0.002 (1), 0.29	
High blood pressure pre-gestation	Yes	0	0	3 (4.6)	0	0	1 (1.6)
	No	34 (100)	40 (100)	62 (100)	76 (100)	64 (100)	60 (98.4)

Study Group N=163 Control Group N=177	X^2 (df), P value	a		3.58 (1), 0.058		1.05 (1), 0.479	
High blood pressure 1st trimester **Study Group N=163 Control Group N=177**	Yes	0	0	4 (6.2)	0	0	2 (3.3)
	No	34 (100)	40 (100)	61 (93.8)	76 (100)	64 (100)	59 (100)
	X^2 (df), P value	a		4.81 (1), 0.028**		0.473	
Diabetes pre-gestation **Study Group N=163 Control Group N=177**	Yes	0	0	0	1 (1.3)	0	0
	No	34 (100)	40 (100)	65 (100)	75 (98.7)	64 (100)	61 (100)
	X^2 (df), P value	a		0.86 (1), 0.353		a	
Diabetes 1st trimester **Study Group N=163 Control Group N=177**	Yes	2 (5.9)	1 (2.5)	0	2 (2.6)	2 (3.1)	0
	No	32 (94.1)	39 (97.5)	65 (100)	74 (97.4)	62 (96.9)	61 (100)
	X^2 (df), P value	0.462		0.188		0.164	
Asthma pre-gestation **Study Group N=162 Control Group N=177**	Yes	0	0	2 (3.1)	0	1 (1.6)	1 (1.6)
	No	34 (100)	40 (100)	63 (96.9)	76 (100)	62 (100)	60 (98.4)
	X^2 (df), P value	a		2.37 (1), 0.124		0.001 (1), 0.822	
Asthma 1st trimester **Study Group N=162 Control Group N=177**	Yes	0	2 (5)	3 (4.6)	1 (1.3)	0	1 (1.6)
	No	34 (100)	38 (95)	62 (95.4)	75 (98.7)	63 (100)	60 (98.4)
	X^2 (df), P value	1.75 (1), 0.186		1.38 (1), 0.239		1.04 (1), 0.308	
Convulsions pre-gestation **Study Group N=163 Control Group N=177**	Yes	0	0	0	0	0	0
	No	34 (100)	40 (100)	65 (100)	76 (100)	64 (100)	61 (100)
	X^2 (df), P value	a		a		a	
Convulsions 1st trimester **Study Group N=163 Control Group N=177**	Yes	0	0	0	0	0	0
	No	34 (100)	40 (100)	65 (100)	76 (100)	64 (100)	61 (100)
	X^2 (df), P value	a		a		a	
Vaginal bleeding **Study Group N=162 Control Group N=177**	Yes	2 (5.9)	1 (2.5)	2 (3.1)	1 (1.3)	7 (11.1)	3 (4.9)
	No	32 (94.1)	39 (97.5)	63 (96.9)	75 (98.7)	56 (88.9)	58 (95.1)
	X^2 (df), P value	0.54 (1), 0.462		0.52 (1), 0.47		1.6 (1), 0.205	
Maternal exposure to X-ray 1st trimester **Study Group N=164 Control Group N=179**	Yes	0	1 (2.5)	3 (4.9)	1 (1.3)	3 (4.9)	0
	No	34 (100)	39 (97.5)	62 (95.4)	76 (98.7)	62 (95.4)	62 (100)
	X^2 (df), P value	0.86 (1), 0.353		1.42 (1), 0.234		2.93 (1), 0.087	
Maternal supplement use							
Folic acid pre-gestation **Study Group N=164 Control Group N=179**	Yes	6 (17.6)	71(2.5)	3 (4.6)	4 (5.2)	7 (10.8)	12 (19.4)
	No	28 (82.4)	39 (97.5)	62 (95.4)	73 (94.8)	58 (89.2)	50 (80.6)
	X^2 (df), P value OR (95% CI)	4.92 (1), 0.026** 0.12 (0.04-0.31)		0.03 (1), 0.874		1.84 (1), 0.175	
Folic acid 1st trimester	Yes	20 (58.8)	28 (70)	37 (56.9)	51 (66.2)	39 (60)	49 (79)

Study Group N=164 **Control Group N=179**	No	14 (41.2)	12 (30)	28 (43.1)	26 (33.8)	26 (40)	13 (21)
	X^2 (df), P value OR (95% CI)	1.01 (1), 0.316		1.3 (1), 0.255		5.4 (1), 0.02** 0.4 (0.18-0.87)	
Multivitamins pre-gestation **Study Group N=164** **Control Group N=179**	Yes	4 (11.2)	0	3 (4.6)	2 (2.6)	3 (4.6)	2 (3.2)
	No	30 (88.2)	40 (100)	62 (95.4)	75 (97.4)	62 (95.4)	60 (96.8)
	X^2 (df), P value	4.98 (1), 0.026		0.42 (1), 0.516		0.16 (1), 0.687	
Multivitamins 1st trimester **Study Group N=181** **Control Group N=179**	Yes	10 (29.4)	9 (22.5)	12 (18.5)	13 (16.9)	11 (17.2)	11 (17.7)
	No	42 (70.6)	31 (77.5)	53 (81.5)	64 (83.1)	53 (82.8)	51 (82.3)
	X^2 (df), P value	0.46 (1), 0.498		0.06 (1), 0.806		0.01 (1), 0.935	
Iron pre-gestation **Study Group N=164** **Control Group N=218**	Yes	4 (11.8)	5 (6.4)	4 (6.2)	1 (5.6)	8 (2.9)	4 (6.2)
	No	30 (88.2)	73 (93.6)	61 (93.8)	75 (97.4)	57 (97.1)	60 (93.8)
	X^2 (df), P value	1.13 (1), 0.288		1.1 (1), 0.294		0.51 (1), 0.477	
Iron 1st trimester **Study Group N=164** **Control Group N=219**	Yes	13 (38.2)	24 (30.8)	23 (51.4)	25 (32.5)	18 (25.7)	26 (40.6)
	No	21 (61.8)	54 (69.2)	42 (64.6)	52 (67.5)	47 (74.3)	38 (59.4)
	X^2 (df), P value	0.61 (1), 0.714		0.71 (1), 0.607		0.14 (1), 0.142	
Calcium 1st trimester **Study Group N=196** **Control Group N=179**	Yes	1 (2.9)	6 (15)	4 (6.2)	9 (11.7)	3 (4.7)	5 (8.1)
	No	66 (97.1)	34 (85)	61 (93.8)	68 (88.3)	61 (95.3)	57 (91.9)
	X^2 (df), P value	3.12 (1), 0.077		1.3 (1), 0.255		0.6 (1), 0.437	
Smoking							
Maternal smoking **Study Group N=162** **Control Group N=179**	Yes	1 (2.9)	1 (2.5)	5 (7.7)	5 (6.5)	0	2 (3.2)
	No	33 (97.1)	39 (97.5)	60 (92.3)	72 (93.5)	63 (100)	60 (96.8)
	X^2 (df), P value	0.014 (1), 0.907		0.08 (1), 0.781		2.06 (1), 0.151	
Paternal smoking **Study Group N=162** **Control Group N=179**	Yes	9 (26.5)	13 (32.5)	25 (38.5)	28 (36.4)	23 (36.5)	22 (35.5)
	No	25 (73.5)	27 (67.5)	40 (61.5)	49 (63.6)	40 (63.5)	40 (64.5)
	X^2 (df), P value	0.32 (1), 0.572		0.07 (1), 0.797		0.01 (1),0.905	
Paternal tobacco **Study Group N=171** **Control Group N=179**	Yes	6 (17.6)	11 (27.5)	19 (29.2)	26 (33.8)	18 (28.6)	16 (25.8)
	No	28 (82.4)	29 (72.5)	46 (70.8)	51 (66.2)	54 (71.4)	46 (74.2)
	X^2 (df), P value	1 (1), 0.315		0.34 (1), 0.563		0,12 (1), 0.728	
Paternal waterpipe **Study Group N=162** **Control Group N=179**	Yes	7 (20.6)	2 (5)	10 (15.4)	3 (3.9)	5 (7.9)	5 (8.1)
	No	27 (79.4)	38 (95)	55 (84.6)	74 (96.1)	58 (92.1)	57 (91.9)
	X^2 (df), P value OR (95% CI)	4.2 (1), 0.041** 4.93 (0.95-25.58)		5.59 (1), 0.018** 4.48 (1.18-17.07)		0.001 (1), 0.979	
Paternal Jorak **Study Group N=162**	Yes	4 (11.8)	0	5 (7.7)	0	4 (6.3)	2 (3.2)
	No	30 (88.2)	40 (100)	60 (92.3)	77 (100)	59 (93.7)	60 (96.8)

Control Group N=179	X^2 (df), P value	0.498 (1), 0.026**		6.14 (1) 0.013**		0.67 (1), 0.414	
Paternal Moasel	Yes	4 (11.8)	2 (5)	6 (9.2)	3 (3.9)	2 (3.2)	4 (6.5)
Study Group N=162	No	30 (88.2)	38 (95)	59 (90.8)	74 (96.1)	61 (96.8)	58 (93.5)
Control Group N=179	X^2 (df), P value	1.13 (1), 0.288		1.69 (1), 0.194		0.73 (1), 0.391	
Maternal passive smoking	Yes	8 (23.5)	4 (10)	15 (23.1)	17 (22.1)	10 (15.9)	11 (17.7)
Study Group N=162	No	26 (76.5)	36 (90)	50 (76.9)	60 (77.9)	53 (84.1)	51 (82.3)
Control Group N=179	X^2 (df), P value	5.25 (2), 0.072		1.45 (2), 0.484		2.62 (2), 0.27	
Maternal stress							
Family problems	Yes	17 (50)	6 (15)	22 (33.8)	24 (31.2)	24 (38.1)	13 (21)
Study Group N=162	No	17 (50)	34 (85)	43 (66.2)	53 (68.8)	39 (61.9)	49 (79)
Control Group N=179	X^2 (df), P value OR (95% CI)	10.51 (1), 0.001** 5.67 (1.89-16.99)		0.12 (1), 0.734		4.39 (1), 0.036** 2.32 (1.05-5.14)	
Mother complains of being under stress	Yes	17 (50)	9 (22.5)	12 (41.5)	29 (37.7)	29 (46)	16 (25.8)
Study Group N=147	No	17 (50)	31 (77.5)	38 (58.5)	48 (62.3)	34 (54)	46 (74.2)
Control Group N=179	X^2 (df), P value OR (95% CI)	6.1 (1), 0.014** 4.22 (1.57-11.36)		0.22 (1), 0.638		5.54 (1), 0.019** 2.45 (1.15-5.21)	
Depression pre-gestation	Yes	0	0	0	1 (1.3)	3 (4.7)	1 (1.6)
Study Group N=163	No	34 (100)	40 (100)	65 (100)	75 (98.7)	61 (95.3)	60 (98.4)
Control Group N=177	X^2 (df), P value	a		0.86 (1), 0.353		0.09 (1), 0.333	
Depression 1st trimester	Yes	2 (5.9)	0	2 (3.1)	2 (2.6)	3 (4.7)	1 (1.6)
Study Group N=163	No	32 (94.1)	40 (100)	63 (96.9)	74 (97.4)	61 (95.3)	60 (98.4)
Control Group N=177	X^2 (df), P value	2.42 (1), 0.12		0.03 (1), 0.874		0.93 (1), 0.333	
Severe morning sickness	Yes	4 (11.8)	2 (5)	8 (12.3)	6 (7.9)	8 (12.7)	4 (6.6)
Study Group N=162	No	30 (88.2)	38 (95)	57 (87.7)	70 (92.1)	55 (87.3)	57 (93.4)
Control Group N=177	X^2 (df), P value	1.13 (1), 0.288		0.76 (1), 0.382		1.33 (1), 0.248	
Threatened abortion	Yes	2 (5.9)	2 (5)	2 (3.1)	0	6 (9.5)	5 (8.2)
Study Group N=162	No	32 (94.1)	38 (95)	63 (96.9)	76 (100)	57 (90.5)	56 (91.8)
Control Group N=177	X^2 (df), P value	0.03 (1), 0.867		2.37 (1), 0.124		0.06 (1), 0.795	
Abdominal pain pre-gestation	Yes	2 (5.9)	0	2 (3.1)	4 (5.3)	2 (3.2)	1 (1.6)
Study Group N=162	No	32 (94.1)	40 (100)	63 (96.9)	72 (94.7)	61 (96.8)	60 (98.4)
Control Group N=177	X^2 (df), P value	0.12		0.521		0.578	
Abdominal pain 1st trimester	Yes	6 (17.6)	2 (5)	6 (9.2)	7 (9.2)	6 (9.5)	2 (3.3)
Study Group N=162	No	28 (82.4)	38 (95)	59 (90.8)	69 (90.8)	57 (90.5)	59 (96.7)
Control Group N=177	X^2 (df), P value	3.05 (1), 0.081		0.000 (1), 0.997		2 (1), 0.157	
Maternal domestic environmental exposure							

Exposure to chemicals pre-gestation **Study Group N=162 Control Group N=177**	Yes	12 (35.3)	10 (25)	22 (33.8)	20 (26.3)	17 (27)	18 (29.5)
	No	22 (64.7)	30 (75)	43 (66.2)	56 (73.7)	46 (73)	43 (70.5)
	X^2 (df), P value	0.93 (1), 0.334		0.95 (1), 0.33		0.09 (1), 0.755	
Exposure to chemicals 1st trimester **Study Group N=162 Control Group N=177**	Yes	10 (29.4)	9 (22.5)	22 (33.8)	22 (28.9)	17 (27)	18 (29.5)
	No	24 (70.6)	31 (77.5)	43 (66.2)	54 (71.1)	46 (73)	43 (70.5)
	X^2 (df), P value	0.46 (1), 0.498		0.39 (1), 0.531		0.1 (1), 0.755	
Exposure to solvents pre-gestation **Study Group N=162 Control Group N=179**	Yes	4 (11.8)	7 (17.5)	10 (15.4)	8 (10.4)	7 (11.1)	8 (12.9)
	No	30 (88.2	33 (82.5)	55 (84.6)	69 (98.6)	56 (88.9)	54 (87.1)
	X^2 (df), P value	0.48 (1), 0.489		0.79 (91)0.373		0.1 (1), 0.758	
Exposure to solvents 1st trimester **Study Group N=162 Control Group N=179**	Yes	2 (5.9)	6 (15)	7 (10.8)	6 (7.8)	7 (11.1)	8 (12.9)
	No	32 (94.1)	34 (85)	58 (89.2)	71 (92.2)	56 (88.9)	54 (87.1)
	X^2 (df), P value	1.59 (1), 0.208		0.38 (), 0.54		0.1 (1), 0.758	
Exposure to pesticides pre-gestation **Study Group N=162 Control Group N=179**	Yes	5 (14.7)	9 (22.5)	9 (13.8)	12 (15.6)	10 (15.9)	12 (19.4)
	No	29 (85.3)	31 (77.5)	56 (86.2)	65 (84.4)	53 (84.1)	50 (80.6)
	X^2 (df), P value	0.73 (1), 0.394		0.09 (1), 0.771		0.26 (1), 0.609	
Exposure to pesticides 1st trimester **Study Group N=131 Control Group N=179**	Yes	5 (14.7)	14 (18.2)	5 (14.7)	5 (12.5)	11 (17.5)	11 (17.7)
	No	29 (85.3)	63 (81.8)	29 (85.3)	35(87.5)	52 (82.5)	51 (82.3)
	X^2 (df), P value	0.08 (1), 0.782		0.002 (1), 0.966		0.002 (1), 0.967	
Exposure to incense pre-gestation **Study Group N=139 Control Group N=193**	Yes	9 (26.5)	13 (32.5)	25 (38.5)	40 (51.9)	13 (32.5)	25 (39.7)
	No	25 (73.5)	40 (57.5)	40 (61.5)	37 (48.1)	27 (67.5)	38 (60.3)
	X^2 (df), P value	0.32 (1), 0.572		2.6 (1), 0.108		1.79 (1), 0.181	
Exposure to incense 1st trimester **Study Group N=139 Control Group N=180**	Yes	9 (26.5)	12 (30)	25 (38.5)	41 (53.2)	12 (30)	25 (39.7)
	No	25 (73.5)	28 (70)	40 (61.5)	36 (46.8)	28 (70)	38 (60.3)
	X^2 (df), P value	0.11 (1), 0.737		3.1 (1), 0.078		2.31 (1), 0.129	
Maternal drinking water	X^2= (df), Pvalue	**8.6 (3), 0.035****		**12.53 (3), 0.006****		**2.64 (3), 0.451**	
Types of maternal drinking water	Tap	8 (23.5)	3 (7.5)	20 (31.2)	16 (21.1)	12 (19.4)	16 (26.7)
	Bottled	23 (67.6)	31 (77.5)	39 (60.9)	50 (65.8)	45 (72.6)	39 (65)

Study Group N=160 Control Group N=176	P value	0.08		0.24		0.33	
	Well	2 (5.9)	0	5 (7.8)	1 (1.3)	3 (4.8)	1 (1.7)
	P value	0.67		0.23		0.67	
	Zamzam	1 (2.9)	6 (15)	0	9 (11.8)	2 (3.2)	4 (6.7)
	P value OR (95% CI)	0.03** 16 (1.32-194.63)		0.033**		0.451	
Consanguinity **Study Group N=157 Control Group N=168**	Yes	15 (42.9)	23 (60.5)	38 (63.3)	33 (47.1)	36 (58.1)	37 (61.7)
	No	20 (57.1)	15 (39.5)	22 (36.7)	37 (52.9)	26 (41.9)	23 (38.3)
	X^2 (df), P value	0.014 (1), 0.131		0.08 (1), 0.065		2.06 (1), 0.685	
Family history **Study Group N=160 Control Group N=175**	Yes	14 (41.2)	10 (25)	24 (36.9)	22 (28.6)	29 (46)	15 (24.2)
	No	19 (55.9)	29 (72.5)	40 (61.5)	53 (68.8)	34 (54)	46 (74.2)
	X^2 (df), P value OR (95% CI)	2.28 (1), 0.32		1.23 (1), 0.54		7.24 (1), 0.027** 2.62 (1.22-5.62)	

Homozygous common allele genotype.
**The Chi-square statistic is significant at the 0.05 level.

[a] Could not analyse because the groups contained zero values.
If one of the cells contained a zero value, the OR and 95% CI were not calculated.

A44: Comparison between cases and controls for maternal IRF6 rs2235371 genotypes in relationship to the different environmental factors including: maternal illness, medication, stress, supplements, paternal smoking and maternal domestic exposure.

Maternal *IRF6* rs2235371 genotype		**CC* (%)**		**TT (%)**		**CT (%)**	
Environmental factors		Study	Control	Study	Control	Study	Control
Maternal medication use and illness							
Antibiotic use pre-gestation **Study Group N=161 Control Group N=179**	Yes	22 (13.7)	8 (4.6)	0	0	0	1 (20)
	No	139 (86.3)	166 (95.4)	0	0	0	4 (80)
	X^2 (df), P value	8.43 (1), 0.004**		a		a	
Antibiotic use 1st trimester **Study Group N=161 Control Group N=179**	Yes	25 (15.5)	17 (9.8)	0	0	0	1 (20)
	No	136 (84.5)	157 (90.2)	0	0	0	4 (80)
	X^2 (df), P value	2.5 (1), 0.112		a		a	
Antipyretic medication pre-gestation **Study Group N=162 Control Group N=179**	Yes	11 (6.8)	17 (9.8)	0	0	0	1 (20)
	No	151 (93.2)	157 (90.2)	0	0	0	4 (80)
	X^2 (df), P value	0.98 (1), 0.323		a		a	
Antipyretic medication 1st trimester **Study Group N=160 Control Group N=176**	Yes	16 (10)	22 (12.1)	0	0	0	3 (60)
	No	144 (90)	149 (87.1)	0	0	0	2 (40)
	X^2 (df), P value	0.67 (1), 0.414		a		a	
Anti-emetic medication pre-gestation **Study Group N=161 Control Group N=179**	Yes	1 (0.6)	0	0	0	0	0
	No	160 (99.4)	174 (100)	0	0	0	5 (100)
	X^2 (df), P value	1.08 (1), 0.289		a		a	
Anti-emetic medication 1st trimester **Study Group N=161 Control Group N=179**	Yes	23 (14.3)	12 (6.9)	0	0	0	2 (40)
	No	138 (85.7)	162 (93.1)	0	0	0	3 (60)
	X^2 (df), P value	4.88 (1), 0.027**		a		a	
Contraceptives pre-	Yes	13 (8.2)	14 (8)	0	0	0	0

gestation **Study Group N=159** **Control Group N=179**	No	146 (91.8)	160 (92)	0	0	0	5 (100)
	X^2 (df), P value	0.002 (1), 0.965		a		a	
Contraceptives 1st trimester **Study Group N=161** **Control Group N=179**	Yes	3 (1.9)	5 (2.9)	0	0	0	0
	No	158 (98.1)	169 (97.1)	0	0	0	5 (100)
	X^2 (df), P value	0.37 (1), 0.545		a		a	
Illness pre-gestation **Study Group N=161** **Control Group N=178**	Yes	45 (28)	17 (9.8)	0	0	0	2 (40)
	No	116 (72)	156 (90.2)	0	0	0	3 (60)
	X^2 (df), P value	18.12 (1), <0.001**		a		a	
Illness 1st trimester **Study Group N=161** **Control Group N=177**	Yes	62 (38.5)	44 (25.6)	0	0	0	3 (60)
	No	99 (61.5)	128 (74.4)	0	0	0	2 (40)
	X^2 (df), P value	6.4 (1) 0.011**		a		a	
Common cold/flu pre-gestation **Study Group N=161** **Control Group N=91**	Yes	33 (20.5)	3 (3.5)	0	0	0	2 (40)
	No	128 (79.5)	83 (96.5)	0	0	0	3 (60)
	X^2 (df), P value	25.45 (1), <0.001**		a		a	
Common cold/flu 1st trimester **Study Group N=161** **Control Group N=177**	Yes	36 (22.4)	23 (13.4)	0	0	0	1 (20)
	No	125 (77.6)	149 (86.6)	0	0	0	4 (80)
	X^2 (df), P value	4.61 (1), 0.032**		a		a	
Fever pre-gestation **Study Group N=161** **Control Group N=177**	Yes	17 (10.6)	6 (3.5)	0	0	0	2 (40)
	No	144 (89.4)	166 (96.5)	0	0	0	3 (60)
	X^2 (df), P value	6.47 (1), 0.011**		a		a	
Fever 1st trimester **Study Group N=161** **Control Group N=177**	Yes	23 (14.3)	14 (8.1)	0	0	0	1 (20)
	No	138 (85.7)	158 (91.9)	0	0	0	4 (80)
	X^2 (df), P value	3.18 (1), 0.075		a		a	
Urinary tract infection pre-gestation **Study Group N=160**	Yes	6 (3.8)	3 (1.7)	0	0	0	0
	No	154 (96.2)	169 (98.3)	0	0	0	5 (100)

Control Group N=177	X^2 (df), P value	1.27 (1), 0.261		a		a	
Urinary tract infection 1st trimester **Study Group N=160 Control Group N=177**	Yes	5 (3.1)	7 (4.1)	0	0	0	1 (20)
	No	155 (96.9)	165 (95.9)	0	0	0	4 (80)
	X^2 (df), P value	0.21 (1), 0.645		a		a	
High blood pressure pre-gestation **Study Group N=161 Control Group N=177**	Yes	3 (1.9)	1 (0.6)	0	0	0	0
	No	158 (98.1)	171 (99.4)	0	0	0	5 (100)
	X^2 (df), P value	1.15 (1), 0.283		a		a	
High blood pressure 1st trimester **Study Group N=161 Control Group N=177**	Yes	4 (2.5)	2 (1.2)	0	0	0	0
	No	157 (97.5)	170 (98.8)	0	0	0	5 (100)
	X^2 (df), P value	0.82 (1), 0.365		a		a	
Diabetes pre-gestation **Study Group N=161 Control Group N=177**	Yes	0 (0)	1 (0.6)	0	0	0	0
	No	161 (100)	171 (99.4)	0	0	0	5 (100)
	X^2 (df), P value	0.94 (1), 0.333		a		a	
Diabetes 1st trimester **Study Group N=161 Control Group N=177**	Yes	4 (2.5)	3 (1.7)	0	0	0	0
	No	157 (97.5)	169 (98.3)	0	0	0	5 (100)
	X^2 (df), P value	0.22 (1), 0.638		a		a	
Asthma pre-gestation **Study Group N=161 Control Group N=177**	Yes	3 (1.9)	1 (0.6)	0	0	0	0
	No	158 (98.1)	171 (99.4)	0	0	0	5 (100)
	X^2 (df), P value	1.17 (1), .28		a		a	
Asthma 1st trimester **Study Group N=161 Control Group N=177**	Yes	3 (1.9)	4 (2.3)	0	0	0	0
	No	158 (98.1)	168 (97.7)	0	0	0	5 (100)
	X^2 (df), P value	0.08, 0.775					
Convulsion pre-gestation **Study Group N=161 Control Group N=177**	Yes	0 (0)	0 (0)	0	0	0	0
	No	161 (100)	172 (100)	0	0	0	5 (100)
	X^2 (df), P value	a		a		a	

Convulsion 1st trimester **Study Group N=161 Control Group N=177**	Yes	0 (0)	0 (0)	0	0	0	0
	No	161 (100)	172 (100)	0	0	0	5 (100)
	X^2 (df), P value	a		a		a	
Vaginal bleeding **Study Group N=160 Control Group N=177**	Yes	11 (6.9)	5 (2.9)	0	0	0	0
	No	149 (93.1)	167 (97.1)	0	0	0	5 (100)
	X^2 (df), P value	0.249		a		a	
Maternal supplement use							
Folic acid pre-gestation **Study Group N=162 Control Group N=179**	Yes	16 (9.9)	16 (9.2)	0	0	0	1 (20)
	No	146 (90.1)	158 (90.8)	0	0	0	4 (80)
	X^2 (df), P value	0.05 (1), 0.832		a		a	
Folic acid 1st trimester **Study Group N=162 Control Group N=179**	Yes	96 (59.3)	124 (71.3)	0	0	0	4 (80)
	No	66 (40.7)	50 (28.7)	0	0	0	1 (20)
	X^2 (df), P value	4.35 (1), 0.021*		a		a	
Multivitamins pre-gestation **Study Group N=162 Control Group N=91**	Yes	10 (5.3)	2 (2.3)	0	0	0	0
	No	152 (94.7)	84 (97.7)	0	0	0	5 (100)
	X^2 (df), P value	3.15 (1), 0.076		a		a	
Multivitamins 1st trimester **Study Group N=161 Control Group N=179**	Yes	31 (19.3)	32 (18.4)	0	0	0	1 (20)
	No	130 (80.7)	142 (81.6)	0	0	0	4 (80)
	X^2 (df), P value	0.04 (1), 0.84		a		a	
Iron pre-gestation **Study Group N=161 Control Group N=179**	Yes	12 (7.5)	9 (5.2)	0	0	0	1 (20)
	No	149 (92.5)	165 (94.8)	0	0	0	4 (80)
	X^2 (df), P value	0.74 (1), 0.39		a		a	
Iron 1st trimester **Study Group N=161 Control Group N=179**	Yes	51 (31.7)	58 (33.3)	0	0	0	3 (60)
	No	110 (68.3)	116 (66.7)	0	0	0	2 (40)
	X^2 (df), P value	0.11 (1), 0.746		a		a	

Calcium 1st trimester **Study Group N=143** **Control Group N=179**	Yes	8 (5)	20 (11.5)	0	0	0	0
	No	135 (95)	154 (88.5)	0	0	0	5 (100)
	X^2 (df), P value	4.65 (1), 0.031**		a		a	
Smoking							
Maternal smoking **Study Group N=151** **Control Group N=179**	Yes	6 (3.8)	7 (4)	0	0	0	1 (20)
	No	145 (96.2)	167 (96)	0	0	0	4 (80)
	X^2 (df), P value	0.02 (1), 0.897		a		a	
Paternal smoking **Study Group N=160** **Control Group N=179**	Yes	57 (35.6)	62 (35.6)	0	0	0	1 (20)
	No	103 (64.4)	112 (64.4)	0	0	0	4 (80)
	X^2 (df), P value	0.000 (1), , 0.999		a		a	
Paternal tobacco **Study Group N=160** **Control Group N=179**	Yes	43 (26.9)	52 (29.9)	0	0	0	1 (20)
	No	117 (73.1)	122 (70.1)	0	0	0	4 (80)
	X^2 (df), P value	0.37 (1), 0.542		a		a	
Paternal water-pipe smoking **Study Group N=160** **Control Group N=** 179	Yes	22	10	0	0	0	0
	No	138	164	0	0	0	5
	X^2 (df), P value	6.16 (1), 0.013**		a		a	
Paternal Jorak **Study Group N=160** **Control Group N=179**	Yes	13 (8.1)	2 (1.1)	0	0	0	0
	No	147 (91.9)	172 (98.9)	0	0	0	5 (100)
	X^2 (df), P value	9.46 (1), 0.002**		a		a	
Paternal Moasel **Study Group N=160** **Control Group N=179**	Yes	12 (7.5)	9 (5.2)	0	0	0	0
	No	148 (92.5)	165 (94.8)	0	0	0	5 (100)
	X^2 (df), P value	0.77 (1), 0.381		a		a	
Maternal passive smoking **Study Group N=160** **Control Group N=179**	Yes	33 (20.6)	31 (17.8)	0	0	0	0
	No	127 (79.4)	143 (82.2)	0	0	0	5 (100)
	X^2 (df), P value	0.43 (1), 0.515		a		a	
Maternal stress							

Mother complains of being under stress **Study Group N=160 Control Group N=179**	Yes	63 (39.4)	42 (24.1)	0	0	0	1 (20)
	No	97 (60.6)	132 (75.9)	0	0	0	4 (80)
	X^2 (df), P value	8.98 (1), 0.003**		a		a	
Depression pre-gestation **Study Group N=161 Control Group N=177**	Yes	73 (45.6)	52 (29.9)	0	0	0	2 (40)
	No	87 (54.4)	122 (70.1)	0	0	0	3 (60)
	X^2 (df), P value	8.82 (1), 0.003**		a		a	
Depression 1st trimester **Study Group N=161 Control Group N=177**	Yes	3 (1.9)	2 (1.2)	0	0	0	0
	No	158 (98.1)	170 (98.8)	0	0	0	5 (100)
	X^2 (df), P value	0.28 (1), 0.599		a		a	
Severe morning sickness **Study Group N=160 Control Group N=177**	Yes	7 (4.3)	2 (1.2)	0	0	0	1 (20)
	No	154 (95.7)	170 (98.8)	0	0	0	4 (80)
	X^2 (df), P value	3.21 (`), 0.073		a		a	
Threatened abortion **Study Group N=160 Control Group N= 168**	Yes	20 (12.5)	11 (6.4)	0	0	0	1 (20)
	No	140 (87.5)	161 (93.6)	0	0	0	4 (80)
	X^2 (df), P value	3.65 (1), 0.056		a		a	
Abdominal pain pre-gestation **Study Group N=160 Control Group N=177**	Yes	10 (6.2)	7 (4.1)	0	0	0	0
	No	150 (93.8)	156 (95.9)	0	0	0	5 (100)
	X^2 (df), P value	0.81 (1), 0.368		a		a	
Abdominal pain 1st trimester **Study Group N=160 Control Group N=177**	Yes	6 (3.8)	5 (2.9)	0	0	0	0
	No	154 (96.2)	167 (97.1)	0	0	0	5 (100)
	X^2 (df), P value	0.18 (1), 0.67		a		a	
Mother complains of being under stress **Study Group N=160 Control Group N=179**	Yes	18 (11.2)	11 (6.4)	0	0	0	0
	No	142 (88.8)	161 (93.6)	0	0	0	5 (100)
	X^2 (df), P value	2.45 (1), 0.974		a		a	
Maternal domestic environmental exposure							

Exposure to chemicals pre-gestation **Study Group N=160 Control Group N=177**	Yes	50 (31.2)	47 (27.3)	0	0	0	1 (20)
	No	110 (68.8)	125 (72.7)	0	0	0	4 (80)
	X^2 (df), P value	0.62 (1), 0.432		a		a	
Exposure to chemicals 1st trimester **Study Group N=160 Control Group N=155**	Yes	48 (30)	26 (27.9)	0	0	0	1 (20)
	No	112 (70)	124 (72.1)	0	0	0	4 (80)
	X^2 (df), P value	0.674		a		a	
Exposure to solvent pre-gestation **Study Group N=160 Control Group N=155**	Yes	20 (10.18 (1), 2.5)	23 (13.2)	0	0	0	0
	No	140 (87.5)	151 (86.6)	0	0	0	5 (100)
	X^2 (df), P value	0.04 (1), 0.845		a		a	
Exposure to solvent 1st trimester **Study Group N=160 Control Group N=179**	Yes	15 (9.4)	20 (11.5)	0	0	0	0
	No	145 (90.6)	154 (88.5)	0	0	0	5 (100)
	X^2 (df), P value	0;.4 (1), 0.528		a		a	
Exposure to pesticides pre-gestation **Study Group N=187 Control Group N=179**	Yes	24 (14.7)	32 (18.4)	0	0	0	1 (20)
	No	163 (85.3)	142 (81.6)	0	0	0	4 (80)
	X^2 (df), P value	0.69 (1), 0.407		a		a	
Exposure to pesticides 1st trimester **Study Group N=160 Control Group N=179**	Yes	28 (17.5)	29 (16.7)	0	0	0	1 (20)
	No	132 (82.5)	145 (83.3)	0	0	0	4 (80)
	X^2 (df), P value	0.04 (1), 0.84		a		a	
Exposure to incense pre-gestation **Study Group N=160 Control Group N=179**	Yes	58 (36.2)	84 (48.3)	0	0	0	1 (20)
	No	102 (63.8)	90 (51.7)	0	0	0	4 (80)
	X^2 (df), P value	4.93 (1), 0.026**		a		a	
Exposure to incense in the 1st trimester **Study Group N=160 Control Group N=179**	Yes	58 (36.2)	85 (48.9)	0	0	0	1 (20)
	No	102 (63.8)	89 (51.1)	0	0	0	4 (80)
	X^2 (df), P value	5.4 (1), 0.02**		a		a	
Maternal exposure to X-	Yes	6 (3.7)	2 (1.1)	0	0	0	0

ray in the 1st trimester **Study Group N=162** **Control Group N=179**	No	156 (96.3)	172 (98.9)	0	0	0	5 (100)
	X^2 (df), P value	2.36 (1), 0.125		a		a	
Type of maternal drinking water **Study Group N=158** **Control Group N=176**	Tap	40 (25.3)	35 (20.5)	0	0	0	0
	Bottled	105 (66.5)	116 (67.8)	0	0	0	4 (80)
	Well	10 (6.3)	2 (1.2)	0	0	0	0
	Zamzam	3 (1.9)	18 (10.5)	0	0	0	1 (20)
	X^2 (df), P value	16.4 (3), 0.001**		a		a	
Consanguinity **Study Group N=156** **Control Group N=168**	Yes	88 (56.4)	91 (55.8)	0	0	0	2 (40)
	No	68 (43.6)	72 (44.2)	0	0	0	3 (60)
	X^2 (df), P value	0.01 (1), 0.917		a		a	
Family history of birth defects **Study Group N=158** **Control Group N=175**	Yes	65 (40.6)	45 (25.2)	0	0	0	2 (40)
	No	93 (58.1)	125 (71.8)	0	0	0	3 (60)
	X^2 (df), P value	8.43 (1), 0.015**		a		a	

**The Chi-square statistic is significant at the 0.05 level

* Homozygous common allele genotype

[a] Not possible to analyse because the groups contain zero values

If one of the cells contains zero value, the OR and 95% CI are not calculated

A45: Relationship between maternal VAX1 rs4752028 genotypes and different environmental factors, including: maternal illness, medication, stress, supplements, paternal smoking and maternal domestic exposure using case-only study design.

Maternal *VAX1* rs4752028		**TT* (%)**	**CC (%)**	**CT (%)**
Maternal medication use and illness				
Antibiotic pre-gestation X^2=8.83, df=2, P= 0.012** **N=162**	Yes	16 (15.5)	4 (36.4)	2 (4.2)
	No	87 (84.5)	7 (63.6)	46 (95.8)
	P value OR (95% CI)		0.097 3.11 (0.81-11.86)	0.062 0.24 (0.05-1.07)
Antibiotic at 1st trimester X^2=1.98, df=2, P= 0.371 **N=162**	Yes	19(18.4)	1 (9.1)	5 (10.4)
	No	84 (81.6)	10 (90.9)	43 (89.6)
	P value OR (95% CI)		0.449 0.44 (0.05-3.67)	0.215 0.51 (0.18-1.47)
Antipyretic medication pre-gestation X^2=0.12, df=2, P= 0.944 **N=163**	Yes	7 (6.7)	1 (9.1)	3 (6.2)
	No	97 (93.3)	10 (90.9)	45 (93.8)
	P value OR (95% CI)		0.771 1.39 (0.15-12.43)	0.991 0.92 (0.23-3.74)
Antipyretic medication 1st trimester X^2=0.041, df=2, P= 0.98 **N=161**	Yes	10 (9.7)	1 (9.1)	5 (10.6)
	No	93 (90.3)	10 (90.9)	42 (89.4)
	P value OR (95% CI)		0.947 0.93 (0.11-8.04)	0.86 1.11 (0.36-3.44)
Anti-emetic medication pre-gestation X^2=0.58, df=2, P=750 **N=162**	Yes	1 (1)	0	0
	No	102 (99)	11 (100)	48 (100)
	P value OR (95% CI)		0.512 a	0.831 A
Anti-emetic medication 1st trimester X^2=3.97, df=2, P= 0.138 **N=162**	Yes	19 (18.4)	2 (18.2)	3 (6.2)
	No	84 (81.6)	9 (81.8)	45 (93.8)
	P value OR (95% CI)		0.983 0.98 (0.2-4.92)	0.059 0.29 (0.08-1.05)
Contraceptives pre-gestation X^2=0.323, df=2, P= 0.851 **N=160**	Yes	9 (8.9)	1 (9.1)	3 (6.2)
	No	92 (91.1)	10 (90.9)	45 (93.8)
	P value OR (95% CI)		0.984 1.02 (0.12-8.92)	0.579 0.68 (0.18-2.64)
Contraceptives 1st trimester X^2=2.06, df=2, P= 0.357 **N=162**	Yes	1 (1)	0	2 (4.2)
	No	102 (99)	11 (100)	46 (95.8)
	P value OR (95% CI)		0.512 a	0.229 0.44 (0.09-2.14)
Illness pre-gestation X^2=1.92, df=2, P= 0.*384* **N=162**	Yes	28 (27.2)	5 (45.5)	12 (25)
	No	75 (72.8)	6 (54.5)	36 (75)
	P value OR (95% CI)		0.213 2.23 (0.63-7.9)	0.777 0.89 (0.41-1.96)
Illness 1st trimester X^2=4.3, df=2, P= 0.116	Yes	43 (41.7)	6 (54.5)	13 (27.1)
	No	60 (58.3)	5 (45.5)	35 (72.9)

N=162	P value OR (95% CI)		0.419 1.67 (0.48-5.84)	0.085 0.52 (0.25-1.09)
Common cold/flu pre-gestation X^2=0.4, df=2, P= 0.818 **N=162**	Yes	21 (20.4)	3 (23.5)	9 (18.8)
	No	82 (79.6)	8 (76.5)	39 (81.2)
	P value OR (95% CI)		0.596 1.46 (0.36-6)	0.814 0.9 (0.38-2.15)
Common cold/flu 1st trimester X^2=1.37, df=2, P= 0.504 **N=162**	Yes	22 (21.4)	4 (36.4)	10 (20.8)
	No	81 (78.6)	7 (63.6)	38 (79.2)
	P value OR (95% CI)		0.268 2.1 (0.56-7.84)	0.941 0.97 (0.42-2.25)
Fever pre-gestation X^2=1.44, df=2, P= 0.487 **N=162**	Yes	13 (12.6)	1 (9.1)	3 (6.2)
	No	90 (87.4)	10 (90.9)	45 (93.8)
	P value OR (95% CI)		0.736 0.69 (0.08-5.86)	0.246 0.46 (0.13-1.7)
Fever 1st trimester X^2=2.5, df=2, P= 0.287 **N=162**	Yes	18 (17.5)	1 (9.1)	4 (8.3)
	No	85 (82.5)	10 (90.9)	44 (91.7)
	P value OR (95% CI)		0.487 0.47 (0.06-3.92)	0.147 0.43 (0.135-1.35)
Urinary tract infection pre-gestation X^2=0.46, df=2, P= 0.794 **N=161**	Yes	4 (3.9)	0	2 (4.2)
	No	98 (96.1)	11 (100)	46 (95.8)
	P value OR (95% CI)		0.974 a	0.943 1.07 (0.19-6.03)
Urinary tract infection 1st trimester X^2=0.54, df=2, P= 0.763 **N=161**	Yes	3 (2.9)	0	2 (4.2)
	No	99 (97.1)	11 (100)	46 (95.8)
	P value OR (95% CI)		0.891 a	0.698 1.4 (0.23-8.88)
High blood pressure pre-gestation X^2=4.08, df=2, P= 0.13 **N=162**	Yes	2 (1.9)	1 (9.1)	0
	No	101 (98.1)	10 (90.9)	48 (100)
	P value OR (95% CI)		0.202 5.05 (0.42-60.7)	0.576
High blood pressure 1st trimester X^2=12.61, df=2, P= 0.002** **N=162**	Yes	2 (1.9)	2 (18.2	0
	No	101 (98.1)	9 (81.8)	48 (100)
	P value OR (95% CI)		0.022** 11.22 (1.41-89.4)	0.576
Diabetes pre-gestation a **N=162**	Yes	0	0	0
	No	103 (100)	11 (100)	48 (100)
	P value OR (95% CI)		a	a
Diabetes 1st trimester X^2=3.3, df=2, P= 0.192	Yes	0	1 (9.1)	0
	No	103 (100)	10 (90.9)	48 (100)

N=162	P value OR (95% CI)		0.042** a	a
Asthma pre-gestation X^2=2.04, df=2, P= 0.361 **N=176**	Yes	1 (1)	0	1 (1.6)
	No	101 (99)	11 (100)	62 (100)
	P value OR (95% CI)		0.516 a	0.732 1.63 (0.1-26.52)
Asthma 1st trimester X^2=2.04, df=2, P= 0.361 **N=176**	Yes	1 (1)	0	1 (1.6)
	No	101 (99)	11 (100)	62 (100)
	P value OR (95% CI)		0.516 a	0.732 1.63 (0.1-26.52)
Convulsions pre-gestation a **N=162**	Yes	0	0	0
	No	103 (100)	11 (100)	48 (100)
	P value OR (95% CI)		a	a
Convulsions 1st trimester a **N=162**	Yes	0	0	0
	No	103 (100)	11 (100)	48 (100)
	P value OR (95% CI)		a	a
Vaginal bleeding X^2=0.8, df=2, P= 0.669 **N=161**	Yes	7 (6.9)	0	3 (6.2)
	No	95 (93.1)	11 (100)	45 (93.8)
	P value OR (95% CI)		0.692	0.888 0.9 (0.22-3.66)
Maternal exposure to X-ray 1st trimester X^2=4.26, df=2, P= 0.119 **N=163**	Yes	2 (1.9)	0	4 (8.3)
	No	102 (98.1)	11 (100)	44 (91.7)
	OR (95% CI)		0.715	0.083 4.64 (0.82-26.25)
Maternal supplement use				
Folic acid pre-gestation X^2=0.03, df=2, P= 0.985 **N=133**	Yes	10 (9.6)	1 (9.1)	5 (10.4)
	No	64 (90.4)	10 (90.9)	43 (89.6)
	P value OR (95% CI)		0.686 0.64 (0.07-5.56)	0.612 0.74 (0.24-2.33)
Folic acid 1st trimester X^2=1.52, df=2, P= 0.467 **N=163**	Yes	60 (57.7)	5 (45.5)	31 (64.6)
	No	44 (42.7)	6 (54.5)	17 (35.4)
	P value OR (95% CI)		0.44 0.61 (0.18-2.13)	0.421 1.34 (0.66-2.71)
Multivitamins pre-gestation X^2=0.78, df=2, P= 0.676 **N=163**	Yes	7 (6.7)	0	3 (6.2)
	No	97 (93.3)	11 (100)	45 (93.8)
	P value OR (95% CI)		0.702 a	0.912 0.92 (0.23-3.74)
Multivitamins 1st trimester X^2=0.17, df=2, P= 0.917	Yes	22 (21.4)	2 (18.2	9 (18.8)
	No	81 (78.6)	9 (81.8)	39 (81.2)

N=162	P value OR (95% CI)		0.806 0.82 (0.16-4.06)	0.712 0.84 (0.36-2.02)
Iron pre-gestation X^2=1.57, df=2, P= 0.456 **N=162**	Yes	7 (6.8)	0	5 (10.4)
	No	96 (93.2)	11 (100)	43 (89.6)
	P value OR (95% CI)		0.697 a	0.447 1.59 (0.48-5.31)
Iron 1st trimester X^2=0.66, df=2, P= 0.718 **N=179**	Yes	34 (33)	4 (36.4)	18 (25.7)
	No	69 (67)	7 (63.6)	47 (74.3)
	P value OR (95% CI)		0.823 1.16 (0.32-4.24)	0.468 0.78 (0.39-1.54)
Calcium 1st trimester X^2=0.81, df=2, P= 0.669 **N=162**	Yes	6 (5.8)	0	2 (4.2)
	No	97 (94.2)	11 (100)	46 (95.8)
	P value OR (95% CI)		0.776a	0.673 0.7 (0.14-3.62)
Smoking				
Maternal smoking X^2=1.08, df=2, P= 0.582 **N=161**	Yes	3 (2.9)	1 (9.1)	2 (4.2)
	No	99 (97.1)	10 (90.9)	46 (95.8)
	P value OR (95% CI)		0.32 0.3 (0.31-34.77)	0.698 1.43 (0.23-8.88)
Paternal smoking X^2=1.95, df=2, P= 0.337 **N=161**	Yes	34 (33.3)	6 (54.5)	17 (35.4)
	No	68 (66.7)	5 (45.5)	31 (64.6)
	P value OR (95% CI)		0.172 2.4 (0.68-8.43)	0.802 0.1 (0.53-2.25)
Paternal tobacco X^2=2.46, df=2, P= 0.293 **N=161**	Yes	30 (29.4)	4 (36.4)	9 (18.8)
	No	72 (70.6)	7 (63.6)	39 (81.2)
	P value OR (95% CI)		0.634 1.37 (90.37-5.03)	0168 0.55 (0.24-1.28)
Paternal waterpipe X^2=2.81, df=2, P= 0.245 **N=161**	Yes	11 (10.8)	3 (27.3)	8 (16.7)
	No	91 (89.2)	8 (72.7)	40 (83.3)
	P value OR (95% CI)		0.13 3.1 (0.72-13.45)	0.316 1.65 (0.62-4.42)
Maternal passive smoking X^2=2.8, df=2, P= 0.246 **N=161**	Yes	22 (21.6)	4 (36.4)	7 (14.6)
	No	80 (78.4)	7 (63.6)	41 (85.4)
	P value OR (95% CI)		0.276 2.08 (0.56-7.75)	0.315 0.62 (0.24-1.57)
Maternal stress				
Family problems X^2=2.73, df=2, P= 0.255 **N=161**	Yes	35 (34.3)	5 (45.5)	23 (47.9)
	No	67 (65.7)	6 (54.5)	25 (52.1)
	P value OR (95% CI)		0.466 1.6 (0.45-5.6)	0.112 1.76 (0.88-3.54)
Mother complains of being under stress	Yes	42 (41.2)	5 (45.5)	26 (54.2)
	No	60 (58.8)	6 (54.5)	22 (45.8)

N=161	P value OR (95% CI)		0.785 1.19 (0.34-4.16)	0.138 1.69 (0.85-3.37)
Depression pre-gestation X^2=1.75, df=2, P= 0.417 **N=162**	Yes	3 (2.9)	0	0
	No	100 (97.1)	11 (100)	48 (100)
	P value OR (95% CI)		0.885 a	0.424 a
Depression 1st trimester X^2=4.19, df=2, P= 0.1.23 **N=162**	Yes	7 (6.8)	0	0
	No	96 (93.2)	11 (100)	48 (100)
	P value OR (95% CI)		0.697 a	0.169 a
Severe morning sickness X^2=1.23, df=2, P= 0.54 **N=161**	Yes	14 (13.7)	2 (18.2)	4 (8.3)
	No	88 (86.3)	9 (81.8)	44 (91.7)
	P value OR (95% CI)		0.688 1.4 (0.27-7.15)	0.348 0.57 (0.18-1.84)
Threatened abortion X^2=0.18, df=2, P= 0.916 **N=161**	Yes	6 (5.9)	1 (9.1)	3 (6.2)
	No	96 (94.1)	10 (90.9)	45 (93.8)
	P value OR (95% CI)		0.678 1.6 (0.17-14.66)	0.93 1.06 (0.26-4.46)
Abdominal pain pre-gestation X^2=2.04, df=2, P= 0.361 **N=124**	Yes	2 (3.1)	0	4 (8.3)
	No	63 (96.9)	11 (100)	44 (91.7)
	P value OR (95% CI)		0.95 a	0.236 2.86 (0.5-16.32)
Abdominal pain 1st trimester X^2=0.662, df=2, P= 0.794 **N=161**	Yes	11 (10.8)	3 (23.5)	2 (4.2)
	No	91 (89.2)	8 (76.5)	46 (95.8)
	P value OR (95% CI)		0.13 3.1 (0.72-13.45)	0.195 0.36 (0.08-1.69)
Maternal domestic environmental exposure				
Exposure to chemicals pre-gestation X^2=0.894, df=2, P= 0.639 **N=161**	Yes	35 (34.3)	3 (23.5)	13 (27.1)
	No	67 (65.7)	8 (76.5)	35 (72.9)
	P value OR (95% CI)		0.64 0.72 (0.18-2.88)	0.377 0.71 (0.33-1.52)
Exposure to chemicals 1st trimester X^2=0.48, df=2, P= 0.785 **N=161**	Yes	33 (32.4)	3 (23.5)	13 (27.1)
	No	69 (97.6)	8 (76.5)	35 (72.9)
	P value OR (95% CI)		0.732 0.78 (0.19-3.15)	0.514 0.78 (0.36-1.66)
Exposure to solvents pre-gestation X^2=2.58, df=2, P= 0.276 **N=161**	Yes	10 (9.8)	2 (18.2	9 (18.8)
	No	92 (90.2)	9 (81.8)	39 (81.2)
	P value OR (95% CI)		0.4 2.04 (0.39-10.81)	0.13 2.12 (0.8-5.63)
Exposure to solvents 1st trimester X^2=3.07, df=2, P=215 **N=161**	Yes	7 (6.9)	2 (18.2	7 (14.6)
	No	95 (93.1)	9 (81.8)	41 (85.4)
	P value		0.207	0.138

	OR (95% CI)		3.02 (0.54-16.74)	2.32 (0.76-7.03)
Exposure to pesticides pre-gestation X^2=3.04, df=2, P=219 **N=161**	Yes	19 (13.8)	1 (9.1)	4 (8.3)
	No	83 (86.2)	10 (90.9)	44 (91.7)
	P value OR (95% CI)		0.443 0.44 (0.05-3.62)	0.112 0.4 (0.13-1.24)
Exposure to pesticides 1st trimester X^2=2.05, df=2, P= 0.358 **N=161**	Yes	21 (20.6)	1 (9.1)	6 (12.5)
	No	81 (79.4)	10 (90.9)	42 (87.5)
	P value OR (95% CI)		0.376 0.39 (0.05-3.18)	0.234 0.55 (0.21-1.47)
Exposure to incense pre-gestation X^2=0.73, df=2, P= 0.693 **N=161**	Yes	39 (38.2)	3 (23.5)	16 (33.3)
	No	63 (61.8)	8 (76.5)	32 (66.7)
	P value OR (95% CI)		0.478 0.61 (0.15-2.42)	0.561 0.81 (0.39-1.66)
Exposure to incense 1st trimester $X^2$0.44=, df=2, P= 0.802 **N=161**	Yes	38 (37.3)	3 (23.5)	17 (35.4)
	No	64 (62.7)	8 (76.5)	31 (64.6)
	P value OR (95% CI)		0.516 0.63 (0.16-2.53)	0.828 0.92 (0.45-1.89)
Type of maternal drinking water X^2=5.21, df=2, P= 0.517 **N=159**	Tap	21 (31.2)	4 (36.4)	15 (31.2)
	Bottled	72 (60.9)	6 (54.5)	28 (58.3)
	P value OR (95% CI)		0.232 2.29 (0.59-8.86)	0.13 1.84 (0.83-4.06)
	Well	6 (7.8)	1 (9.1)	3 (6.2)
	P value OR (95% CI)		0.91 1.14 (0.11-12.24)	0.649 1.43 (0.31-6.64)
	Zamzam	1 (0.07)	0	2 (4.2)
	P value OR (95% CI)		0.786 a	0.418 0.36 (0.03-4.31)
Consanguinity X^2=1.41, df=2, P= 0.495 **N=156**	Yes	57 (58.2)	8 (66.7)	23 (50)
	No	41 (41.8)	4 (33.3)	23 (50)
	P value OR (95% CI)		0.573 1.44 (0.41-5.1)	0.359 0.72 (0.36-1.45)
Family history X^2=6.18, df=2, P= 0.186 **N=159**	Yes	46 (45.1)	5 (45.5)	16 (33.3)
	No	56 (54.9)	6 (54.5)	30 (62.5)
	P value OR (95% CI)		0.982 1.01 (0.29-3.54)	0.241 0.65 (0.32-1.34)

* Homozygous common allele genotype

**The Chi-square statistic is significant at the 0.05 level

[a] Not possible to analyse because the groups contain zero values

If one of the cells contains zero value, the OR and 95% CI are not calculated

A46: Maternal *VAX1* rs4752028 allele frequency in relationship to different environmental factors including: maternal illness, medication, stress, supplements, paternal smoking and maternal domestic exposure using case-only study design.

Maternal VAX1 rs4752028		**T* (%)**	**C (%)**
Maternal medication use and illness			
Antibiotic pre-gestation **N=324**	Yes	34 (13.4)	10 (14.3)
	No	220 (86.6)	60 (85.7)
	X^2 (df), P value OR (95% CI)	0.04 (1) 0.846 1.08 (0.5-2.3)	
Antibiotic at 1st trimester **N=324**	Yes	43 (16.9)	7 (10.0)
	No	211(83.1)	63 (90.0)
	X^2 (df), P value OR (95% CI)	2.02 (1) 0.16 0.55 (0.23-1.27)	
Antipyretic medication pre-gestation **N=326**	Yes	17 (6.6)	5 (7.14)
	No	239 (93.4)	65 (92.9)
	X^2 (df), P value OR (95% CI)	0.02 (1) 0.882 1.08 (0.38-3.04)	
Antipyretic medication 1st trimester **N=322**	Yes	25 (9.9)	7 (10.1)
	No	228 (90.1)	62 (89.9)
	X^2 (df), P value OR (95% CI)	0.00 (1) 0.948 1.03 (0.43-2.49)	
Anti-emetic medication pre-gestation **N=324**	Yes	2 (0.8)	0 (0.0)
	No	252 (99.2)	70 (100.0)
	X^2 (df), P value	0.55 (1) 0.46	
Anti-emetic medication 1st trimester **N=324**	Yes	41 (16.1)	7 (10.0)
	No	213 (83.9)	63 (90.0)
	X^2 (df), P value OR (95% CI)	1.64 (1) 0.205 0.58 (0.25-1.35)	
Contraceptives pre-gestation **N=320**	Yes	21 (8.4)	5 (7.1)
	No	229 (91.6)	65 (92.9)
	X^2 (df), P value OR (95% CI)	0.12 (1) 0.734 0.84 (0.3-2.31)	
Contraceptives 1st trimester **N=324**	Yes	4 (1.57)	2 (2.9)
	No	250 (98.4)	68 (97.1)
	X^2 (df), P value OR (95% CI)	0.50 (1) 0.487 1.84 (0.33-10.25)	
Illness pre-gestation **N=324**	Yes	68 (26.8)	22 (31.4)
	No	186 (73.2)	48 (68.6)

	X^2 (df), P value OR (95% CI)	0.59 (1) 0.442 1.25 (0.7-2.23)	
Illness 1st trimester **N=324**	Yes	99 (39.0)	25 (35.7)
	No	155 (61.0)	45 (64.3)
	X^2 (df), P value OR (95% CI)	0.25 (1) 0.619 0.87 (0.5-1.51)	
Common cold/flu pre-gestation **N=324**	Yes	51 (20.1)	15 (21.4)
	No	203 (79.9)	55 (78.6)
	X^2 (df), P value OR (95% CI)	0.06 (1) 0.80 1.09 (0.57-2.08)	
Common cold/flu 1st trimester **N=324**	Yes	54 (21.3)	18 (25.7)
	No	200 (78.7)	52 (74.3)
	X^2 (df), P value OR (95% CI)	0.63 (1) 0.428 1.28 (0.69-2.37)	
Fever pre-gestation **N=324**	Yes	29 (11.4)	5 (7.1)
	No	225 (88.6)	65 (92.9)
	X^2 (df), P value OR (95% CI)	1.07 (1) 0.306 0.6 (0.22-1.6)	
Fever 1st trimester **N=468**	Yes	184 (42.2)	6(8.6)
	No	214 (53.8)	64 (91.4)
	X^2 (df), P value OR (95% CI)	35.01 (1) <0.001** 0.11 (0.05-0.26)	
Urinary tract infection pre-gestation **N=322**	Yes	10 (4.0)	2 (2.9)
	No	242 (96 .0)	68 (97.1)
	X^2 (df), P value OR (95% CI)	0.19 (1) 0.66 0.71 (0.15-2.22)	
Urinary tract infection 1st trimester **N=322**	Yes	8 (3.2)	2 (2.9)
	No	244 (96.8)	68 (97.1)
	X^2 (df), P value OR (95% CI)	0.01 (1) 0.89 0.918 (0.26-3.23)	
High blood pressure pre-gestation **N=324**	Yes	4 (1.6)	2 (2.9)
	No	250 (98.4)	68 (97.1)
	X^2 (df), P value OR (95% CI)	0.49 (1) 0.48 0.987 (0.19-4.32)	
High blood pressure 1st trimester **N=324**	Yes	4 (1.6)	4 (5.7)
	No	250 (98.4)	66 (94.3)
	X^2 (df), P value OR (95% CI)	3.90 (1) 0.05** 3.77 (0.92-15.55)	
Diabetes pre-gestation **N=324**	Yes	0	0
	No	254 (100.0)	70 (100.0)
	X^2 (df), P value	a	
Diabetes 1st trimester	Yes	0	2 (2.9)

N=324	No	254 (100.0)	68 (97.1)
	X^2 (df), P value	7.30 (1) 0.01**	
Asthma pre-gestation **N=352**	Yes	3 (1.1)	1 (1.2)
	No	264 (98.9)	84 (98.8)
	X^2 (df), P value OR (95% CI)	0.00 (1) 0.968 1.05 (0.11-10.21)	
Asthma 1st trimester **N=248**	Yes	8 (4.5)	2 (2.9)
	No	170 (95.5)	68 (97.1)
	X^2 (df), P value OR (95% CI)	0.35 (1) 0.56 0.91 (0.19-4.37)	
Convulsions pre-gestation **N=324**	Yes	0 (0.0)	0 (0.0)
	No	254 (100.0)	70 (100.0)
	X^2 (df), P value OR (95% CI)	a	
Convulsions 1st trimester **N=324**	Yes	0 (0.0)	0 (0.0)
	No	254 (100.0)	70 (100.0)
	X^2 (df), P value	a	
Vaginal bleeding **N=322**	Yes	17 (6.8)	3 (4.3)
	No	235 (93.3)	67 (95.7)
	X^2 (df), P value OR (95% CI)	0.57 (1) 0.455 0.62 (0.18-2.18)	
Maternal supplement use			
Folic acid pre-gestation **N=266**	Yes	25 (0.8)	7 (10.0)
	No	171 (87.2)	63 (90.0)
	X^2 (df), P value OR (95% CI)	0.37 (1) 0.544 0.76 (0.31-1.84)	
Folic acid 1st trimester **N=326**	Yes	151 (59.0)	41 (58.6)
	No	105 (41.0)	29 (41.4)
	X^2 (df), P value OR (95% CI)	0.00(1) 0.95 0.98 (0.57-1.68)	
Multivitamins pre-gestation **N=326**	Yes	17 (6.6)	3 (4.3)
	No	239 (93.4)	67 (95.7)
	X^2 (df), P value OR (95% CI)	0.53 (1) 0.471 0.63 (0.18-2.21)	
Multivitamins 1st trimester **N=324**	Yes	53 (20.9)	13 (18.6)
	No	201 (79.1)	57 (81.4)
	X^2 (df), P value OR (95% CI)	0.18 (1) 0.673 0.86 (0.44-1.7)	
Iron pre-gestation **N=324**	Yes	19 (7.5)	5 (7.1)
	No	235 (92.5)	65 (92.9)
	X^2 (df), P value OR (95% CI)	0.01 (1) 0.92 0.95 (0.34-2.65)	

Iron 1st trimester **N=358**	Yes	86 (31.7)	26 (29.9)
	No	185 (68.3)	61 (70.1)
	X^2 (df), P value OR (95% CI)	0.10 (1) 0.746 0.92 (0.54-1.55)	
Calcium 1st trimester **N=324**	Yes	14 (5.5)	2 (2.9)
	No	240 (94.5)	68 (97.1)
	X^2 (df), P value OR (95% CI)	0.82 (1) 0.36 0.5 (0.11-2.27)	
Smoking			
Maternal smoking **N=322**	Yes	8 (3.2)	4 (5.7)
	No	244 (96.8)	66 (94.3)
	X^2 (df), P value OR (95% CI)	0.98 (1) 0.328 1.85 (0.54-6.33)	
Paternal smoking **N=322**	Yes	85 (33.7)	29 (41.4)
	No	167 (66.3)	41 (58.6)
	X^2 (df), P value OR (95% CI)	1.42 (1) 0.235 1.39 (0.81-2.39)	
Paternal tobacco **N=322**	Yes	69 (27.4)	17 (24.3)
	No	183 (72.6)	53 (75.7)
	X^2 (df), P value OR (95% CI)	0.27 (1) 0.605 0.85 (0.46-1.57)	
Paternal waterpipe **N=322**	Yes	30 (11.9)	14 (20.0)
	No	222 (88.1)	56 (80.0)
	X^2 (df), P value OR (95% CI)	3.04 (1) 0.085 1.85 (0.92-3.72)	
Maternal passive smoking **N=322**	Yes	51 (20.4)	15 (21.4)
	No	201 (79.8)	55 (78.6)
	X^2 (df), P value OR (95% CI)	0.05 (1) 0.827 1.07 (0.56-2.06)	
Maternal stress			
Family problems **N=322**	Yes	93 (36.9)	33 (41.4)
	No	159 (63.1)	37 (52.9)
	X^2 (df), P value OR (95% CI)	2.41 (1) 0.122 1.52 (0.89-2.6)	
Mother complains of being under stress **N=322**	Yes	110 (43.7)	36 (51.4)
	No	142 (56.4)	34 (48.6)
	X^2 (df), P value OR (95% CI)	1.34 (1) 0.25 1.37 (0.8-2.32)	
Depression pre-gestation **N=324**	Yes	6 (2.4)	0 (0.0)
	No	248 (97.6)	70 (100.0)
	X^2 (df), P value	1.68 (1) 0.19	
Depression 1st trimester	Yes	14 (5.5)	0 (0.0)

N=324	No	240 (94.5)	70 (100.0)
	X^2 (df), P value	4.03 (1) 0.04**	
Severe morning sickness **N=322**	Yes	32 (12.7)	8 (11.4)
	No	220 (87.3)	62 (88.6)
	X^2 (df), P value OR (95% CI)	0.08 (1) 0.776 0.89 (0.39-2.02)	
Threatened abortion **N=322**	Yes	15 (6.0)	5 (7.1)
	No	237 (94.1)	65 (92.9)
	X^2 (df), P value OR (95% CI)	0.13 (1) 0.715 1.22 (0.43-3.46)	
Abdominal pain pre-gestation **N=248**	Yes	8 (4.5)	4 (5.7)
	No	170 (95.5)	66 (94.3)
	X^2 (df), P value OR (95% CI)	0.16 (1) 0.688 1.29 (0.38-4.42)	
Abdominal pain 1st trimester **N=322**	Yes	24 (9.5)	8 (11.4)
	No	228 (90.5)	62 (88.6)
	X^2 (df), P value OR (95% CI)	0.22 (1) 0.638 1.23 (0.53-2.86)	
Maternal domestic environmental exposure			
Exposure to chemicals pre-gestation **N=322**	Yes	83 (32.9)	19 (27.1)
	No	169 (67.1)	51 (72.9)
	X^2 (df), P value OR (95% CI)	0.85 (1) 0.358 0.76 (0.42-1.37)	
Exposure to chemicals 1st trimester **N=322**	Yes	79 (31.4)	19 (27.1)
	No	173 (68.7)	51 (72.9)
	X^2 (df), P value OR (95% CI)	0.46 (1) 0.499 0.82 (0.45-1.47)	
Exposure to solvents pre-gestation **N=322**	Yes	29 (11.5)	13 (18.6)
	No	223 (88.5)	57 (81.4)
	X^2 (df), P value OR (95% CI)	2.41 (1) 0.124 1.75 (0.86-3.59)	
Exposure to solvents 1st trimester **N=322**	Yes	21 (8.3)	11 (15.7)
	No	231 (91.7)	59 (84.3)
	X^2 (df), P value OR (95% CI)	3.33 (1) 0.072 2.05 (0.94-4.49)	
Exposure to pesticides pre-gestation **N=322**	Yes	42 (16.7)	6 (8.6)
	No	210 (83.3)	64 (91.4)
	X^2 (df), P value OR (95% CI)	2.83 (1) 0.099 0.47 (0.19-1.15)	
Exposure to pesticides 1st trimester **N=322**	Yes	48 (19.1)	8 (11.4)
	No	204 (81.0)	62 (88.6)
	X^2 (df), P value	2.21 (1) 0.141	

	OR (95% CI)	0.55 (0.25-1.22)	
Exposure to incense pre-gestation **N=322**	Yes	94 (37.3)	22 (31.4)
	No	158 (62.7)	48 (68.6)
	X^2 (df), P value OR (95% CI)	0.82 (1) 0.37 0.79 (0.45-1.39)	
Exposure to incense 1st trimester **N=322**	Yes	93 (36.9)	23 (32.7)
	No	159 (63.1)	47 (67.1)
	X^2 (df), P value OR (95% CI)	0.39 (1) 0.533 0.84 (0.48-1.47)	
Maternal exposure to X-ray 1st trimester **N=326**	Yes	8 (3.1)	4 (5.7)
	No	248 (96.9)	66 (94.3)
	X^2 (df), P value OR (95% CI)	1.04 (1) 0.315 1.88 (0.55-6.43)	
Type of maternal drinking water X^2=2.5, df=1 and P= 0.47 **N=323**	Tap	57 (23)	23 (30.7)
	Bottled	172 (69.4)	45 (60)
	P value OR (95% CI)	2.13 (1) 0.147 1.54 (0.86-2.77)	
	Well	15 (6)	5 (6.7)
	P value OR (95% CI)	0.739 1.21 (0.39-3.72)	
	Zamzam	4 (1.6)	2 (2.7)
	P-value OR (95% CI)	0.076 0.2 (0.03-1.18)	
Consanguinity **N=312**	Yes	137 (56.6)	39 (55.7)
	No	105 (43.4)	31 (44.3)
	X^2 (df), P value OR (95% CI)	0.02 (1) 0.893 0.96 (0.56-1.65)	
Family history **N=317**	Yes	108 (43.2)	25 (38.2)
	No	142 (56.8)	42 (61.8)
	X^2 (df), P value OR (95% CI)	0.75 (1) 0.387 0.78 (0.45-1.36)	

* Homozygous common allele genotype

**The Chi-square statistic is significant at the 0.05 level

[a] Not possible to analyse because the groups contain zero values

If one of the cells contains zero value, the OR and 95% CI are not calculated

A47: Comparison between cases and controls for maternal *VAX1* rs4752028 genotypes in relationship to different environmental factors including: maternal illness, medication, stress, supplements, paternal smoking and maternal domestic exposure.

Maternal VAX1 rs4752028		**TT* (%)**		**CC (%)**		**CT (%)**	
Environmental factors		Study	Control	Study	Control	Study	Control
Maternal medication use and illness							
Antibiotic use pre-gestation **Study Group N=162 Control Group N=177**	Yes	16 (15.5)	7 (4.9)	4 (36.4)	1 (33.3)	2 (4.2)	1 (3.3)
	No	87 (84.5)	137 (95.1)	7 (63.6)	2 (66.7)	46 (95.8)	29 (96.7)
	X^2 (df), P value OR (95% CI)	8.1 (1), 0.004** 3.6 (1.42-9.1)		0.009 (1), 0.923		0.04 (1), 0.852	
Antibiotic use 1st trimester **Study Group N=162 Control Group N=177**	Yes	19(18.4)	15 (10.4)	1 (9.1)	0	5 (10.4)	3 (10)
	No	84 (81.6)	129 (89.6)	10 (90.9)	3 (100)	43 (89.6)	27 (90)
	X^2 (df), P value OR (95% CI)	3.26 (1), 0.071 1.95 (0.94-4.04)		0.29 (1), 0.588		0.003 (1), 0.953	
Antipyretic medication pre-gestation **Study Group N=163 Control Group N=177**	Yes	7 (6.7)	14 (9.7)	1 (9.1)	1 (33.3)	3 (6.2)	3 (10)
	No	97 (93.3)	130 (90.3)	10 (90.9)	2 (66.7)	45 (93.8)	27 (90)
	X^2 (df), P value	0.7 (1), 0.404		1.13 (1), 0.287		0.37 (1), 0.545	
Antipyretic medication 1st trimester **Study Group N=161 Control Group N=206**	Yes	10 (9.7)	20 (14.1)	1 (9.1)	2 (66.7)	5 (10.6)	11 (18)
	No	93 (90.3)	122 (85.9)	10 (90.9)	1 (33.3)	42 (89.4)	50 (82)
	X^2 (df), P value	1.06 (1), 0.302 1.07		4.64 (1), 0.063 (0.002-1.18)		0.008 (1), 0.929	
Anti-emetic medication pre-gestation **Study Group N=162 Control Group N=177**	Yes	1 (1)	0	0	0	0	0
	No	102 (99)	144 (100)	11 (100)	3 (100)	48 (100)	30 (100)
	X^2 (df), P value	1.4 (1), 0.236					
Anti-emetic medication 1st trimester **Study Group N=162 Control Group N=177**	Yes	19 (18.4)	12 (8.3)	2 (18.2)	1 (33.3)	3 (6.2)	1 (3.3)
	No	84 (81.6)	132 (91.7)	9 (81.8)	2 (66.7)	45 (93.8)	29 (96.7)
	X^2 (df), P value OR (95% CI)	5.6 (1), 0.018* 2.49 (1.15-5.39)		0.32 (1), 0.571		0.32 (1), 0.57	
Contraceptives pre-	Yes	9 (8.9)	13 (9)	1 (9.1)	1 (33.3)	3 (6.2)	0

gestation **Study Group N=160** **Control Group N=177**	No	92 (91.1)	131 (91)	10 (90.9)	2 (66.7)	45 (93.8)	30 (100)
	X^2 (df), P value	0.001 (1), 0.975		1.13 (1), 0.287		1.95 (1), 0.163	
Contraceptives 1st trimester **Study Group N=162** **Control Group N=177**	Yes	1 (1)	5 (3.5)	0	0	2 (4.2)	0
	No	102 (99)	139 (96.5)	11 (100)	3 (100)	46 (95.8)	30 (100)
	X^2 (df), P value	1.59 (1), 0.208				1.28 (1), 0.257	
Illness pre-gestation **Study Group N=162** **Control Group N=176**	Yes	28 (27.2)	11 (7.7)	5 (45.5)	1 (33.3)	12 (25)	7 (23.3)
	No	75 (72.8)	132 (92.3)	6 (54.5)	2 (66.7)	36 (75)	23 (76.7)
	X^2 (df), P value OR (95% CI)	17.05 (1), <0.001** 4.48 (2.11-9.51)		0.14 (1), 0.707		0.03 (1), 0.868	
Illness 1st trimester **Study Group N=162** **Control Group N=175**	Yes	43 (41.7)	37 (26.1)	6 (54.5)	2 (66.7)	13 (27.1)	8 (26.7)
	No	60 (58.3)	105 (73.9)	5 (45.5)	1 (33.3)	35 (72.9)	22 (73.3)
	X^2 (df), P value OR (95% CI)	6.68 (1), 0.01** 2.03 (1.18-3.5)		0.14 (1), 0.707		0.002 (1), 0.968	
Common cold/flu pre-gestation **Study Group N=162** **Control Group N=175**	Yes	21 (20.4)	5 (3.5)	3 (23.5)	0	9 (18.8)	2 (6.7)
	No	82 (79.6)	137 (96.5)	8 (76.5)	3 (100)	39 (81.2)	28 (93.3)
	X^2 (df), P value OR (95% CI)	17.9 (1), <0.001** 7.02 (2.55-19.32)		1.04 (1), 0.308		2.22 (1), 0.136	
Common cold/flu 1st trimester **Study Group N=162** **Control Group N=175**	Yes	22 (21.4)	21 (14.8)	4 (36.4)	0	10 (20.8)	3 (10)
	No	81 (78.6)	121 (85.2)	7 (63.6)	3 (100)	38 (79.2)	27 (90)
	X^2 (df), P value	1.78 (1), 0.182		1.53 (1), 0.217		1.56 (1), 0.212	
Fever pre-gestation **Study Group N=162** **Control Group N=175**	Yes	13 (12.6)	7 (4.9)	1 (9.1)	0	3 (6.2)	1 (3.3)
	No	90 (87.4)	135 (95.1)	10 (90.9)	3 (100)	45 (93.8)	29 (96.7)
	X^2 (df), P value OR (95% CI)	4.71 (1), 0.03** 2.79 (1.07-7.25)		0.29 (1), 0.588		0.32 (1), 0.57	
Fever 1st trimester **Study Group N=163** **Control Group N=175**	Yes	19 (17.5)	13 (9.2)	1 (9.1)	0	4 (8.3)	2 (6.7)
	No	85 (82.5)	129 (90.8)	10 (90.9)	3 (100)	44 (91.7)	28 (93.3)
	X^2 (df), P value	3.74 (1), 0.053		0.29 (1), 0.588		0.07 (1), 0.788	
Urinary tract infection pre-gestation **Study Group N=161** **Control Group N=175**	Yes	4 (3.9)	2 (1.4)	0	0	2 (4.2)	2 (6.7)
	No	98 (96.1)	140 (98.6)	11 (100)	3 (100)	46 (95.8)	28 (93.3)
	X^2 (df), P value	1.56 (1), 0.211		a		0.23 (1), 0.626	
Urinary tract infection 1st trimester **Study Group N=161** **Control Group N=175**	Yes	3 (2.9)	5 (3.5)	0	0	2 (4.2)	2 (6.7)
	No	99 (97.1)	137 (96.5)	11 (100)	3 (100)	46 (95.8)	28 (93.3)
	X^2 (df), P value	0.06 (1), 0.808		a		0.24 (1), 0.626	
High blood pressure	Yes	2 (1.9)	1 (0.7)	1 (9.1)	0	0	0

pre-gestation **Study Group N=162 Control Group N=175**	No	101 (98.1)	141 (99.3)	10 (90.9)	3 (100)	48 (100)	30 (100)
	X^2 (df), P value	0.76 (1), 0.385		0.29 (1), 0.588		a	
High blood pressure 1st trimester **Study Group N=162 Control Group N=175**	Yes	2 (1.9)	1 (0.7)	2 (18.2	1 (33.3)	0	0
	No	101 (98.1)	141 (99.3)	9 (81.8)	2 (66.7)	48 (100)	30 (100)
	X^2 (df), P value	0.76 (1), 0.385		0.32 (1), 0.57		a	
Diabetes pre-gestation **Study Group N=162 Control Group N=175**	Yes	0	1 (0.7)	0	0	0	0
	No	103 (100)	141 (99.3)	11 (100)	3 (100)	48 (100)	30 (100)
	X^2 (df), P value	0.73 (1), 0.393		a		a	
Diabetes 1st trimester **Study Group N=162 Control Group N=175**	Yes	0	3 (2.1)	1 (9.1)	0	0	0
	No	103 (100)	139 (97.9)	10 (90.9)	3 (100)	48 (100)	30 (100)
	X^2 (df), P value	0.16 (1), 0.689		0.29 (1), 0.588		a	
Asthma pre-gestation **Study Group N=176 Control Group N=175**	Yes	1 (1)	0	0	1 (33.3)	1 (1.6)	0
	No	101 (99)	142 (100)	11 (100)	2 (66.7)	62 (100)	30 (100)
	X^2 (df), P value	1.4 (1), 0.237		3.95 (1), 0.047**		1.28 (1), 0.257	
Asthma 1st trimester **Study Group N=124 Control Group N=175**	Yes	3 (4.6)	3 (2.1)	0	0	2 (4.2)	1 (3.3)
	No	62 (95.4)	139 (97.9)	11 (100)	3 (100)	46 (95.8)	29 (96.7)
	X^2 (df), P value	0.47 (1), 0.492		a		0.04 (1), 0.852	
Convulsions pre-gestation **Study Group N=162 Control Group N=175**	Yes	0	0	0	0	0	0
	No	103 (100)	142 (100)	11 (100)	3 (100)	48 (100)	30 (100)
	X^2 (df), P value	a		a		a	
Convulsions 1st trimester **Study Group N=162 Control Group N=175**	Yes	0	0	0	0	0	0
	No	103 (100)	142 (100)	11 (100)	3 (100)	48 (100)	30 (100)
	X^2 (df), P value	a		a		a	
Vaginal bleeding **Study Group N=161 Control Group N=175**	Yes	7 (6.9)	5 (3.5)	0	0	3 (6.2)	0
	No	95 (93.1)	137 (96.5)	11 (100)	3 (100)	45 (93.8)	30 (100)
	X^2 (df), P value	1.42 (1), 0.234		a		1.95 (1), 0.163	
Maternal exposure to X-ray in the 1st trimester **Study Group N=163 Control Group N=177**	Yes	2 (1.9)	2 (1.3)	0	0	4 (8.3)	0
	No	102 (98.1)	142 (98.7)	11 (100)	3 (100)	44 (91.7)	30 (100)
	X^2 (df), P value	0.11 (1), 0.742		a		2.6 (1), 1.05	
Maternal supplement use							
Folic acid pre-gestation	Yes	10 (9.6)	13 (9)	1 (9.1)	0	5 (10.4)	4 (13.3)
	No	64 (90.4)	131 (91)	10 (90.9)	3 (100)	43 (89.6)	26 (86.7)

Study Group N=133 Control Group N=177	X^2 (df), P value	0.03 (1), 0.875		0.29 (1), 0.588		0.15 (1), 0.695	
Folic acid 1st trimester **Study Group N=163 Control Group N=156**	Yes	60 (57.7)	103 (71.5)	5 (45.5)	2 (66.7)	31 (64.6)	7 (76.7)
	No	44 (42.7)	41 (28.5)	6 (54.5)	1 (33.3)	17 (35.4)	2 (23.3)
	X^2 (df), P value OR (95% CI)	5.13 (1), 0.023** 0.54 (0.32-0.92)		0.42 (1), 0.515		1.27 (1), 0.261	
Multivitamins pre-gestation **Study Group N=163 Control Group N=177**	Yes	7 (6.7)	2 (1.4)	0	0	3 (6.2)	2 (6.7)
	No	97 (93.3)	142 (98.6)	11 (100)	3 (100)	45 (93.8)	28 (93.3)
	X^2 (df), P value OR (95% CI)	4.93 (1), 0.044** 5.12 (1.04-25.19)		a		0.005 (1), 0.942	
Multivitamins 1st trimester **Study Group N=162 Control Group N=177**	Yes	22 (21.4)	26 (18.1)	2 (18.2	0	9 (18.8)	7 (23.3)
	No	81 (78.6)	118 (81.9)	9 (81.8)	3 (100)	39 (81.2)	23 (76.7)
	X^2 (df), P value	0.42 (1), 0.518		0.64 (1), 0.425		0.24 (1), 0.626	
Iron pre-gestation **Study Group N=162 Control Group N=177**	Yes	7 (6.8)	8 (5.6)	0	1 (33.3)	5 (10.4)	1 (3.3)
	No	96 (93.2)	136 (94.4)	11 (100)	2 (66.7)	43 (89.6)	29 (96.7)
	X^2 (df), P value	0.16 (1), 0.687		3.95 (1), 0.139		1.31 (1), 0.253	
Iron 1st trimester **Study Group N=179 Control Group N=110**	Yes	34 (33)	25 (32.5)	4 (36.4)	0	18 (25.7)	11 (36.7)
	No	69 (67)	52 (67.5)	7 (63.6)	3 (100)	47 (74.3)	19 (63.3.4)
	X^2 (df), P value	0.42 (1), 0.779		0.64 (1), 0.217		0.24 (1), 0.372	
Calcium 1st trimester **Study Group N=162 Control Group N=177**	Yes	6 (5.8)	14 (9.7)	0	0	2 (4.2)	6 (20)
	No	97 (94.2)	130 (90.3)	11 (100)	3 (100)	46 (95.8)	24 (80)
	X^2 (df), P value	1.22 (1), .268		a		5.03 (1), 0.025** 0.17 (0.033-0.93)	
Smoking							
Maternal smoking **Study Group N=161 Control Group N=177**	Yes	3 (2.9)	5 (3.5)	1 (9.1)	0	2 (4.2)	3 (10)
	No	99 (97.1)	139 (96.5)	10 (90.9)	3 (100)	46 (95.8)	27 (90)
	X^2 (df), P value	0.005 (1), 0.817		0.29 (1), 0.588		1.05 (1), 0.306	
Paternal smoking **Study Group N=161 Control Group N=177**	Yes	34 (33.3)	54 (37.5)	6 (54.5)	0	17 (35.4)	9 (30)
	No	68 (66.7)	90 (62.5)	5 (45.5)	3 (100)	31 (64.6)	21 (70)
	X^2 (df), P value	0.45 (1), 0.502		2.86 (1), 0.091		0.24 (1), 0.622	
Paternal tobacco **Study Group N=161 Control Group N=177**	Yes	30 (29.4)	44 (30.6)	4 (36.4)	0	9 (18.8)	9 (30)
	No	72 (70.6)	100 (69.4)	7 (63.6)	3 (100)	39 (81.2)	21 (70)
	X^2 (df), P value	0.04 (1), 0.847		1.53 (1), 0.217		1.32 (1), 0.251	
Paternal waterpipe smoking **Study Group N=161 Control Group**	Yes	11 (10.8)	10 (6.9)	3 (27.3)	0	8 (16.7)	0
	No	91 (89.2)	134 (93.1)	8 (72.7)	3 (100)	40 (83.3)	30 (100)
	X^2 (df), P value	1.13 (1), 0.288		1.04 (1), 0.308		5.57 (1), 0.018**	

N=177							
Paternal Jorak **Study Group N=161 Control Group N=177**	Yes	7 (6.9)	2 (1.4)	1 (9.1)	0	5 (10.4)	0
	No	95 (93.1)	142 (98.6)	10 (90.9)	3 (100)	43 (89.6)	30 (100)
	X^2 (df), P value OR (95% CI)	5.08 (1), 0.024** 5.23 (1.06-25.73)		0.29 (1), .588		3.34 (1), 0.068	
Paternal Moasel **Study Group N=161 Control Group N=177**	Yes	6 (5.9)	9 (6.2)	2 (18.2	0	4 (8.3)	0
	No	96 (94.1)	135 (93.8)	9 (81.8)	3 (100)	44 (91.7)	30 (100)
	X^2 (df), P value	0.01 (1), 0.905		0.64 (1), 0.425		2.64 (1), 0.105	
Maternal passive smoking **Study Group N=161 Control Group N=177**	Yes	22 (21.6)	26 (18.1)	4 (36.4)	0	7 (14.6)	6 (20)
	No	80 (78.4)	118 (81.9)	7 (63.6)	3 (100)	41 (85.4)	24 (80)
	X^2 (df), P value	0.47 (1), 0.493		1.53 (1), 0.217		0.39 (1), 0.532	
Maternal stress							
Family problems **Study Group N=162 Control Group N=177**	Yes	35 (34.3)	34 (23.6.2)	5 (45.5)	0	23 (47.9)	8 (26.7)
	No	67 (65.7)	110 (76.4)	6 (54.5)	3 (100)	25 (52.1)	22 (73.3)
	X^2 (df), P value	3.39 (1), 0.066		2.12 (1), 0.145		3.48 (1), 0.062	
Mother complains of being under stress **Study Group N=161 Control Group N=177**	Yes	42 (41.2)	42 (29.2)	5 (45.5)	0	26 (54.2)	11 (36.7)
	No	60 (58.8)	102 (70.8)	6 (54.5)	3 (100)	22 (45.8)	19 (63.3.4)
	X^2 (df), P value OR (95% CI)	3.83 (1), 0.05** 1.7 (1-2.9)		2.12 (1), 0.145		2.27 (1), 0.132	
Depression pre-gestation **Study Group N=162 Control Group N=175**	Yes	3 (2.9)	2 (1.4)	0	0	0	0
	No	100 (97.1)	140 (98.6)	11 (100)	3 (100)	48 (100)	30 (100)
	X^2 (df), P value	0.68 (1), 0.411		a		a	
Depression 1st trimester **Study Group N=162 Control Group N=175**	Yes	7 (6.8)	2 (1.4)	0	0	0	1 (3.3)
	No	96 (93.2)	140 (98.6)	11 (100)	3 (100)	48 (100)	29 (96.7)
	X^2 (df), P value OR (95% CI)	4.9 (1), 0.027** 5.1 (1.04-25.1)		a		1.62 (1), 0.203	
Severe morning sickness **Study Group N=161 Control Group N=175**	Yes	14 (13.7)	9 (6.3)	2 (18.2	0	4 (8.3)	3 (10)
	No	88 (86.3)	133 (93.7)	9 (81.8)	3 (100)	44 (91.7)	27 (90)
	X^2 (df), P value	3.79 (91), 0.051		0.64 (1), 0.425		0.06 (1), 0.802	
Threatened abortion **Study Group N=161 Control Group N=175**	Yes	6 (5.9)	4 (2.8)	1 (9.1)	1 (33.3)	3 (6.2)	2 (6.7)
	No	96 (94.1)	138 (97.2)	10 (90.9)	2 (66.7)	45 (93.8)	28 (93.3)
	X^2 (df), P value	1.42 (1), 0.234		1.13 (1), 0.287		0.005 (1), 0.942	
Abdominal pain pre-gestation **Study Group N=124**	Yes	2 (3.1)	4 (2.8)	0	0	4 (8.3)	1 (3.3)
	No	63 (96.9)	138 (97.2)	11 (100)	3 (100)	44 (91.7)	29 (96.7)

Control Group N=175	X^2 (df), P value	0.23 (1), 0.633				0.04 (1), 0.852	
Abdominal pain 1st trimester **Study Group N=161 Control Group N=175**	Yes	11 (10.8)	8 (5.6)	3 (23.5)	1 (33.3)	2 (4.2)	1 (3.3)
	No	91 (89.2)	134 (94.4)	8 (76.5)	2 (66.7)	46 (95.8)	29 (96.7)
	X^2 (df), P value	2.19 (1), 0.139		0.04 (1), 0.837		0.07 (1), 0.788	
Maternal domestic environmental exposure							
Exposure to chemicals pre-gestation **Study Group N=161 Control Group N=126**	Yes	35 (34.3)	37 (26.1)	3 (23.5)	1 (33.3)	13 (27.1)	7 (23.3)
	No	67 (65.7)	56 (73.9)	8 (76.5)	2 (66.7)	35 (72.9)	23 (76.7)
	X^2 (df), P value	1.95 (1), 0.163		5.09 (1), 0.837		0.14 (1), 0.712	
Exposure to chemicals 1st trimester **Study Group N=161 Control Group N=126**	Yes	33 (32.4)	37 (26.1)	3 (23.5)	1 (33.3)	13 (27.1)	8 (26.7)
	No	69 (97.6)	56 (73.9)	8 (76.5)	2 (66.7)	35 (72.9)	22 (73.3)
	X^2 (df), P value	1.15 (1), 0.284		5.09 (1), 0.837		0.002 (1), 0.968	
Exposure to solvent pre-gestation **Study Group N=161 Control Group N=177**	Yes	10 (9.8)	18 (12.5)	2 (18.2	1 (33.3)	9 (18.8)	3 (10)
	No	92 (90.2)	126 (87.5)	9 (81.8)	2 (66.7)	39 (81.2)	27 (90)
	X^2 (df), P value	0.43 (1), 0.512		0.32 (1), 0.571		1.09 (1), 0.297	
Exposure to solvent 1st trimester **Study Group N=161 Control Group N=177**	Yes	7 (6.9)	17 (11.8)	2 (18.2	1 (33.3)	7 (14.6)	1 (3.3)
	No	95 (93.1)	127 (88.2)	9 (81.8)	2 (66.7)	41 (85.4)	29 (96.7)
	X^2 (df), P value	1.66 (1|), 0.198		0.32 (1), 0.571		2.58 (1), 0.111	
Exposure to pesticides pre-gestation **Study Group N=161 Control Group N=177**	Yes	19 (13.8)	26 (18.1)	1 (9.1)	1 (33.3)	4 (8.3)	6 (20)
	No	83 (86.2)	118 (81.9)	10 (90.9)	2 (66.7)	44 (91.7)	24 (80)
	X^2 (df), P value	0.01 (1), 0.909		1.13 (1), 0.287		2.25 (1), 0.134	
Exposure to pesticides 1st trimester **Study Group N=161 Control Group N=177**	Yes	21 (20.6)	22 (15.3)	1 (9.1)	1 (33.3)	6 (12.5)	7 (23.3)
	No	81 (79.4)	122(84.7)	10 (90.9)	2 (66.7)	42 (87.5)	23 (76.7)
	X^2 (df), P value	1.17 (1), 0.28		1.13 (1), 0.287		1.56 (1), 0.212	
Exposure to incense pre-gestation **Study Group N=161 Control Group N=177**	Yes	39 (38.2)	67 (46.5)	3 (23.5)	3 (100)	16 (33.3)	13 (43.3)
	No	63 (61.8)	77 (53.5)	8 (76.5)	0	32 (66.7)	17 (56.7)
	X^2 (df), P value	1.67 (1), 0.196		5.09 (1), 0.024**		0.79 (1), 0.374	
Exposure to incense in the 1st trimester	Yes	38 (37.3)	69 (47.9)	3 (23.5)	3 (100)	17 (35.4)	12 (40)
	No	64 (62.7)	75 (52.1)	8 (76.5)	0	31 (64.6)	18 (60)

Study Group N=161 Control Group N=177	X^2 (df), P value	2.76 (1), 0.097		5.09 (1), 0.024**		0.17 (1), 0.684	
Type of maternal drinking water	X^2 (df), P value	**16.23 (3), 0.001****		**0.34 (2), 0.844**		**2.82 (3), 0.42**	
Type of maternal drinking water **Study Group N=159 Control Group N=174**	Tap	21 (31.2)	29 (20.6)	4 (36.4)	1 (33.3)	15 (31.2)	5 (16.7)
	Bottled	72 (60.9)	93 (66)	6 (54.5)	2 (66.7)	28 (58.3)	23 (76.7)
	P value	0.838		0.835		0.125	
	Well	6 (7.8)	1 (0.7)	1 (9.1)	0	3 (6.2)	1 (3.3)
	P value	0.059		a		1	
	Zamzam	1 (0.07)	18 (12.8)	0	0	2 (4.2)	1 (3.3)
	X^2 (df), P value OR (95% CI)	0.016** 0.07 (0.01-0.62)		a		0.76	
Consanguinity **Study Group N=156 Control Group N=166**	Yes	57 (58.2)	77 (57)	8 (66.7)	1 (33.3)	23 (50)	14 (50)
	No	41 (41.8)	58 (43)	4 (33.3)	2 (66.7)	23 (50)	14 (50)
	X^2 (df), P value	0.29 (1), 0.864		1.11 (1), 0.292		0.000 (1), 1	
Family history **Study Group N=159 Control Group N=173**	Yes	46 (45.1)	39 (27.9)	5 (45.5)	1 (33.3)	16 (33.3)	6 (20)
	No	56 (54.9)	101 (70.1)	6 (54.5)	2 (66.7)	30 (62.5)	24 (80)
	X^2 (df), P value OR (95% CI)	10.6 (2), 0.005** 2.13 (1.24-3.64)		0.14 (1), 0.707		3.23 (2), 0.199	

**The Chi-square statistic is significant at the 0.05 level

* Homozygous common allele genotype

[a] Not possible to analyse because the groups contain zero values

If one of the cells contains zero value, the OR and 95% CI are not calculated

A48: Relationship between maternal VAX 7078160 genotypes and different environmental factors, including: maternal illness, medication, stress, supplements, paternal smoking and maternal domestic exposure using case-only study design.

Maternal VAX_7078160		**GG**** (%)	AA (%)	AG (%)
Maternal medication use and illness				
Antibiotic pre-gestation X^2=5.13, df=2, P= 0.077 **N=162**	Yes	18 (14.8)	3 (30.0)	1 (3.3)
	No	104 (85.2)	7 (70.0)	29 (96.7)
	P value OR (95% CI)		0.219 2.48 (0.59-10)	0.124 0.2 (0.03-1.56)
Antibiotic at 1st trimester X^2=2.63, df=2, P= 0.269 **N=162**	Yes	22 (18.0)	1 (10.0)	2 (6.7)
	No	100 (82.0)	9 (90.0)	28 (93.3)
	P value OR (95% CI)		0.527 0.51 (0.06-4.19)	0.143 0.32 (0.07-1.47)
Antipyretic medication pre-gestation X^2=1.15, df=2, P= 0.562 **N=163**	Yes	8 (6.6)	0 (0.0)	3 (9.7)
	No	114 (93.4)	10 (100.0)	28 (90.3)
	P value OR (95% CI)		0.766 a	0.551 1.53 (0.8-6.13)
Antipyretic medication 1st trimester X^2=0.033, df=2, P= 0.999 **N=161**	Yes	12 (10.0)	1 (10.0)	3 (9.7)
	No	108 (90.0)	9 (90.0)	28 (90.3)
	P value OR (95% CI)		1 1 (0.12-8.59)	0.957 0.96 (0.25-3.65)
Anti-emetic medication pre-gestation X^2=0.33, df=2, P=848 **N=162**	Yes	1 (0.8)	0 (0.0)	0 (0.0)
	No	121 (99.2)	10 (100.0)	30 (100.0)
	P value OR (95% CI)		0.417 a	0.863 a
Anti-emetic medication 1st trimester X^2=2.03, df=2, P= 0.362 **N=162**	Yes	20 (16.4)	2 (20.0)	2 (6.7)
	No	102 (83.6)	8 (80.0)	28 (93.3)
	P value OR (95% CI)		0.769 1.28 (0.25-6.46)	0.191 0.36 (0.08-1.65)
Contraceptives pre-gestation X^2=1.03, df=2, P= 0.597 **N=160**	Yes	10 (8.3)	0 (0.0)	3 (10.0)
	No	110 (91.7)	10 (100.0)	27 (90.0)
	P value OR (95% CI)		0.641 a	0.772 1.22 (0.31-4.75)
Contraceptives 1st trimester X^2=0.58, df=2, P= 0.748 **N=162**	Yes	2 (1.6)	0 (0.0)	1 (3.3)
	No	120 (98.4)	10 (100.0)	29 (96.7)
	P value OR (95% CI)		0.6 a	0.558 2.22 (0.2-25.37)

Illness pre-gestation X^2=2.54, df=2, P=*280* **N=162**	Yes	30 (24.6)	4 (40.0)	11 (36.7)
	No	92 (75.4)	6 (60.0)	19 (63.3)
	P value OR (95% CI)		0.29 2.04 (0.54-7.7)	0.19 1.78 (0.76-4.15)
Illness 1st trimester X^2=0.63, df=2, P= 0.729 **N=162**	Yes	46 (37.7)	5 (50.0)	11 (36.7)
	No	76 (62.3)	5 (50.0)	19 (63.3)
	P value OR (95% CI)		0.446 1.65 (0.45-6.02)	0.916 0.96 (0.42-2.19)
Common cold/flu pre-gestation X^2=2.13, df=2, P= 0.345 **N=162**	Yes	22 (18.0)	2 (20.06)	9 (30.0)
	No	100 (82.0)	8 (80.0)	21 (70.0)
	P value OR (95% CI)		0.877 1.14 (0.23-5.72)	0.15 1.95 (0.79-4.83)
Common cold/flu 1st trimester X^2=2.06, df=2, P= 0.357 **N=162**	Yes	25 (20.5)	4 (40.0)	7 (23.3)
	No	97 (79.5)	6 (60.0)	23 (76.7)
	P value OR (95% CI)		0.164 2.59 (0.68-9.87)	0.733 1.28 (0.46-3.06)
Fever pre-gestation X^2=0.32, df=2, P= 0.854 **N=162**	Yes	12 (9.8)	1 (10.0)	4 (13.3)
	No	110 (90.2)	9 (90.0)	26 (86.7)
	P value OR (95% CI)		0.987 1.02 (0.12-8.75)	0.578 1.4 (0.42-4.73)
Fever 1st trimester X^2=0.74, df=2, P= 0.69 **N=162**	Yes	18 (14.8)	2 (20.0)	3 (10.0)
	No	104(85.2)	8 (80.0)	27 (90.0)
	P value OR (95% CI)		0.658 1.44 (0.28-7.36)	0.502 0.64 (0.18-2.34)
Urinary tract infection pre-gestation X^2=2.31, df=2, P= 0.314 **N=161**	Yes	5 (4.1)	1 (10.0)	0 (0.0)
	No	116 (95.9)	9 (90.0)	30 (100.0)
	P value OR (95% CI)		0.41 2.58 (0.27-24.5)	0.478 a
Urinary tract infection 1st trimester X^2=1.71, df=2, P= 0.426 **N=161**	Yes	5 (4.1)	0 (0.0)	0 (0.0)
	No	116 (95.9)	10 (100)	30 (100.0)
	P value OR (95% CI)	0.93	0.996 a	0.478 a
High blood pressure pre-gestation X^2=4.25, df=2, P= 0.119 **N=162**	Yes	2 (1.6)	1 (10.0)	0 (0.0)
	No	120 (98.4)	9 (90.0)	30 (100.0)
	P value OR (95% CI)		6.67 (0.55-80.75)	a
High blood pressure 1st trimester **N=162**	Yes	2 (1.6)	2 (20.0)	0 (0.0)
	No	120 (98.4)	8 (80.0)	30 (100.0)
	P value OR (95% CI)		0.011** 15 (1.86-120.86)	0.88 a
High blood pressure 1st	Yes	2 (1.6)	2 (20.0)	0 (0.0)

trimester X^2=13.87, df=2, P= 0.001** **N=162**	No	120 (98.4)	8 (80.0)	30 (100.0)
	P value OR (95% CI)		0.011** 15 (1.86-120.86)	0.88 a
Diabetes pre-gestation a **N=162**	Yes	0 (0.0)	0 (0.0)	0 (0.0)
	No	122 (100.0)	10 (100.0)	30 (100.0)
	P value OR (95% CI)		a	a
Diabetes 1st trimester X^2=3.12, df=2, P= 0.211 **N=162**	Yes	3 (2.5)	1 (10.0)	0 (0.0)
	No	119 (97.5)	9 (90.0)	30 (100.0)
	P value OR (95% CI)		0.219 4.41 (0.42-46.79)	0.704 a
Asthma pre-gestation X^2=4.69, df=2, P= 0.096 **N=161**	Yes	1 (0.8)	0 (0.0)	2 (6.7)
	No	120 (99.2)	10 (100.0)	28 (93.3)
	P value OR (95% CI)		0.42 a	0.084 8.57 (0.75-97.9)
Asthma 1st trimester X^2=4.69, df=2, P= 0.096 **N=161**	Yes	1 (0.8)	0 (0.0)	2 (6.7)
	No	120 (99.2)	10 (100.0)	28 (93.3)
	P value OR (95% CI)		0.42 a	0.084 8.57 (0.75-97.9)
Convulsions pre-gestation a **N=162**	Yes	0 (0.0)	0 (0.0)	0 (0.0)
	No	122 (100.0)	10 (100.0)	30 (100.0)
	P value OR (95% CI)		a	a
Convulsions 1st trimester a **N=161**	Yes	8 (6.6)	0 (0.0)	2 (6.7)
	No	113 (93.4)	10 (100.0)	28 (93.3)
	P value OR (95% CI)		0.761 a	0.991 1 (0.2-5.02)
Maternal exposure to X-ray 1st trimester X^2=1.11, df=2, P= 0.574 **N=163**	Yes	4 (3.3)	0 (0.0)	2 (6.5)
	No	118 (96.7)	10 (100.0)	29 (93.5)
	P value OR (95% CI)		0.882 a	0.798 2.03 (0.36-11.65)
Maternal supplement use				
Folic acid pre-gestation **N=162**	Yes	10 (8.2)	2 (20.0)	4 (12.9)
	No	112 (91.8)	8 (80.0)	26 (87.1)
	P value OR (95% CI)		0.229 2.8 (0.52-15.01)	0.388 1.72 (0.5-5.93)
Folic acid 1st trimester X^2=0.72, df=2, P= 0.697 **N=163**	Yes	72 (59.0)	7 (70.0)	17 (54.8)
	No	50 (41.0)	3 (30.0)	14 (45.2)
	P value OR (95% CI)		0.499 1.62 (0.4-6.57)	0.67 0.84 (0.38-1.87)

Multivitamins pre-gestation X^2=3.43, df=2, P= 0.18 **N=163**	Yes	6 (4.9)	0 (0.0)	4 (12.9)
	No	116 (95.1)	10 (100.0)	27 (87.1)
	P value OR (95% CI)		0.916 a	0.122 2.86 (0.76-10.86)
Multivitamins 1st trimester X^2=0.61, df=2, P= 0.737 **N=162**	Yes	24 (19.7)	3 (30.0)	6 (20.0)
	No	98 (80.3)	7 (70.0)	24 (80.0)
	P value OR (95% CI)		0.441 1.75 (0.42-7.27)	0.968 1.02 (0.38-2.77)
Iron pre-gestation X^2=8.84, df=2, P= 0.012** **N=162**	Yes	6 (4.9)	0 (0.0)	6 (20.0)
	No	116 (95.1)	10 (100.0)	24 (80.0)
	P value OR (95% CI)		0.916a	0.011** 4.83 (1.44-16.27)
Iron 1st trimester X^2=1.86, df=2, P= 0.395 **N=162**	Yes	36 (29.5)	5 (50.0)	10 (33.3)
	No	86 (70.5)	5 (50.0)	20 (66.7)
	P value OR (95% CI)		0.189 2.39 (0.65-8.76)	0.683 1.19 (0.51-2.8)
Calcium 1st trimester X^2=0.85, df=2, P= 0.654 **N=162**	Yes	7 (5.7)	0 (0.0)	1 (3.3)
	No	115 (94.3)	10 (100.0)	29 (96.7)
	P value OR (95% CI)		0.836 a	0.602 0.57 (0.07-4.79)
Smoking				
Maternal smoking X^2=1.17, df=2, P= 0.557 **N=161**	Yes	4 (3.3)	0 (0.0)	2 (6.7)
	No	117 (96.7)	10 (100.0)	28 (93.3)
	P value OR (95% CI)		0.886 a	0.408 2.09 (0.36-11.98)
Paternal smoking X^2=3.65, df=2, P= 0.161 **N=161**	Yes	43 (35.5)	6 (60.0)	8 (26.7)
	No	78 (64.5)	4 (40.0)	22 (73.3)
	P value OR (95% CI)		0.137 2.72 (0.73-10.17)	0.36 0.66 (0.27-1.61)
Paternal tobacco X^2=3.95, df=2, P= 0.139 **N=161**	Yes	35 (28.9)	4 (40.0)	4 (13.3)
	No	86 (71.7)	6 (60.0)	26 (86.7)
	P value OR (95% CI)		0.465 1.64 (0.44-6.16)	0.09 0.38 (0.12-1.16)
Paternal waterpipe X^2=6.98, df=2, P= 0.03** **N=161**	Yes	13 (10.7)	4 (40)	5 (16.7)
	No	108 (89.3)	6 (60)	25 (83.3)
	P value OR (95% CI)		0.016** 5.54 (1.38-22.23)	0.374 1.66 (0.54-5.09)
Maternal passive smoking X^2=3.28, df=2, P= 0.194 **N=161**	Yes	25 (20.7)	4 (40.0)	4 (13.3)
	No	96 (79.3)	6 (60.0)	26 (86.7)

	P value OR (95% CI)		0.169 2.56 (0.67-9.77)	0.366 0.59 (0.19-1.85)
Maternal stress				
Family problems X^2=2.64, df=2, P= 0.267 **N=161**	Yes	43 (35.5)	5 (50.0)	15 (50.0)
	No	78 (64.5)	5 (50.0)	15 (50.0)
	P value OR (95% CI)		0.367 1.81 (0.5-6.62)	0.148 1.81 (0.81-4.06)
Mother complains of being under stress X^2=3.21, df=2, P= 0.201 **N=161**	Yes	50 (41.3)	6 (60.0)	17 (56.7)
	No	71 (58.7)	4 (40.0)	13 (43.3)
	P value OR (95% CI)		0.26 2.13 (0.57-7.94)	0.133 1.86 (0.83-4.16)
Depression pre-gestation X^2=0.58, df=2, P= 0.748 **N=162**	Yes	2 (1.6)	0 (0.0)	1 (3.3)
	No	120 (98.4)	10 (100.0)	29 (96.7)
	P value OR (95% CI)		0.6 a	0.558 2.07 (0.18-23.61)
Depression 1st trimester X^2=0.63, df=2, P= 0.731 **N=162**	Yes	6 (4.9)	0 (0.0)	1 (3.3)
	No	116 (95.1)	10 (100.0)	29 (96.7)
	P value OR (95% CI)		0.916 a	0.712 0.67 (0.08-5.76)
Severe morning sickness X^2=1.95, df=2, P= 0.377 **N=161**	Yes	13 (10.7)	1 (10)	6 (20)
	No	108 (89.3)	9 (90)	24 (80)
	P value OR (95% CI)		0.942 0.92 (0.11-7.88)	0.178 2.07 (0.72-6.02)
Threatened abortion X^2=0.706, df=2, P= 0.702 **N=161**	Yes	8 (6.6)	0 (0.0)	2 (6.7)
	No	113 (93.4)	10 (100.0)	28 (93.3)
	P value OR (95% CI)		0.761 a	0.991 1.01 (0.2-5.02)
Abdominal pain pre-gestation X^2=1.17, df=2, P= 0.557 **N=161**	Yes	4 (3.3)	0 (0.0)	2 (6.7)
	No	117 (96.7)	10 (100.0)	28 (93.3)
	P value OR (95% CI)		0.886 a	0.408 2.09 (0.36-11.98)
Abdominal pain 1st trimester X^2=2.23, df=2, P= 0.329 **N=161**	Yes	11 (9.1)	2 (20.0)	5 (16.7)
	No	110 (90.9)	8 (80.0)	25 (83.3)
	P value OR (95% CI)		0.282 2.5 (0.47-13.27)	0.235 2 (0.64-6.27)
Maternal domestic environmental exposure				
Exposure to chemicals pre-gestation	Yes	37 (30.6)	2 (20.0)	12 (40.0)
	No	84 (69.4)	80 (80.0)	18 (60.0)

X^2=1.66, df=2, P= 0.437 **N=161**	P value OR (95% CI)		<0.001** 0.06 (0.01-0.24)	1.5 (0.66-3.46)
Exposure to chemicals 1st trimester X^2=0.64, df=2, P= 0.728 **N=161**	Yes	37 (30.6)	2 (20.0)	10 (33.3)
	No	84 (69.4)	8 (80.0)	20 (66.7)
	P value OR (95% CI)		0.487 0.57 (0.11-2.8)	0.771 1.14 (0.48-2.66)
Exposure to solvents pre-gestation X^2=2.27, df=2, P= 0.321 **N=161**	Yes	13 (10.7)	2 (20.0)	6 (20.0)
	No	108 (89.3)	8 (80.0)	24 (80.0)
	P value OR (95% CI)		0.386 2.08 (0.4-10.85)	0.178 2.07 (0.72-6.02)
Exposure to solvents 1st trimester X^2=1.9, df=2, P= 0.387 **N=161**	Yes	10 (8.3)	2 (20.0)	4 (13.3)
	No	111 (91.7)	8 (80.0)	26 (86.7)
	P value OR (95% CI)		0.234 2.78 (0.52-14.88	0.396 1.71 (0.5-5.88)
Exposure to pesticides pre-gestation X^2=2.37, df=2, P= 0.306 **N=161**	Yes	21 (17.4)	1 (10.0)	2 (6.7)
	No	100 (82.6)	9 (90.0)	28 (93.3)
	P value OR (95% CI)		0.556 0.53 (0.06-4.4)	0.162 0.34 (0.07-1.54)
Exposure to pesticides 1st trimester X^2=2.02, df=2, P= 0.364 **N=161**	Yes	24 (19.8)	1 (10.0)	3 (10.0)
	No	97 (80.2)	9 (90.0)	27 (90.0)
	P value OR (95% CI)		0.449 0.45 (0.05-3.72)	0.218 0.45 (0.13-1.61)
Exposure to incense pre-gestation X^2=2, df=2, P= 0.367 **N=161**	Yes	47 (38.8)	2 (20.0)	9 (30.0)
	No	74 (61.2)	8 (80.0)	21 (70.0)
	P value OR (95% CI)		0.251 0.39 (0.08-1.93)	0.371 0.67 (0.28-1.6)
Exposure to incense 1st trimester X^2=1.42, df=2, P= 0.492 **N=143**	Yes	46 (38.0)	2 (20.0)	10 (83.3)
	No	75 (62.0)	8 (80.0)	2 (16.7)
	P value OR (95% CI)		0.269 0.41 (0.08-2)	0.008** 8.15 (1.71-38.87)
Type of maternal drinking water X^2=5.68, df=2, P= 0.46	Tap	31 (26.1)	4 (40.0)	5 (16.7)
	Bottled	80 (67.2)	6 (60.0)	20 (66.7)
	P value OR (95% CI)		0.424 1.72 (0.45-6.51)	0.42 0.65 (0.22-1.87)
	Well	6 (5.0)	0 (0.0)	4 (13.3)

N=159	P value OR (95% CI)		0.69 a	0.078 0.24 (0.05-1.17)
	Zamzam	2 (1.7)	0 (0.0)	1 (3.3)
	P value OR (95% CI)		0.836 a	0.39 0.32 (0.02-4.26)
Consanguinity X^2=5.66, df=2, P= 0.059 **N=156**	Yes	69 (59.5)	8 (72.7)	11 (37.9)
	No	47 (40.5)	3 (27.3)	18 (62.1)
	P value OR (95% CI)		0.396 1.82 (0.46-7.2)	0.04** 0.42 (0.18-0.96)
Family history X^2=1.91, df=2, P= 0.753 **N=159**	Yes	49 (40.8)	4 (40.0)	14 (48.3)
	No	71 (59.2)	6 (60.0)	15 (51,7)
	P value OR (95% CI)		0.96 0.97 (0.26-3.6)	0.468 1.35 (0.6-3.05)

**The Chi-square statistic is significant at the 0.05 level

* Homozygous common allele genotype

[a] Not possible to analyse because the groups contain zero values

If one of the cells contains zero value, the OR and 95% CI are not calculated

A49: Maternal *VAX1* 7078160 allele frequency in relationship to different environmental factors including: maternal illness, medication, stress, supplements, paternal smoking and maternal domestic exposure using case-only study design.

Maternal VAX_7078160		G* (%)	A (%)
Maternal medication use and illness			
Antibiotic pre-gestation **N=324**	Yes	37 (13.5)	7 (14.0)
	No	237 (86.5)	43 (86.0)
	X^2 (df), P value OR (95% CI)	0.01 (1) 0.925 1.04 (0.44-2.49)	
Antibiotic at 1st trimester **N=324**	Yes	46 (16.8)	4 (8.0)
	No	228 (83.2)	46 (92.0)
	X^2 (df), P value OR (95% CI)	2.50 (1) 0.11 0.43 (0.15-1.26)	
Antipyretic medication pre-gestation **N=326**	Yes	19 (6.9)	3 (5.9)
	No	256 (93.1)	48 (94.1)
	X^2 (df), P value OR (95% CI)	0.07 (1) 0.79 0.85 (0.24-2.99)	
Antipyretic medication 1st trimester **N=328**	Yes	27 (9.9)	5 (9.1)
	No	246 (90.1)	50 (90.9)
	X^2 (df), P value OR (95% CI)	0.03 (1) 0.855 0.91 (0.33-2.48)	
Anti-emetic medication pre-gestation **N=324**	Yes	2 (0.7)	0.(0.0)
	No	272 (99.3)	50 (100.0)
	X^2 (df), P value OR (95% CI)	0.37 (1) 0.54	
Anti-emetic medication 1st trimester **N=324**	Yes	42 (15.3)	6 (12)
	No	232 (84.7)	44 (88.0)
	X^2 (df), P value OR (95% CI)	0.37 (1) 0.544 0.75 (0.3-1.88)	
Contraceptives pre-gestation **N=320**	Yes	23 (8.5)	3 (6.0)
	No	247 (91.5)	47 (94.0)
	X^2 (df), P value OR (95% CI)	0.36 (1) 0.552 0.69 (0.2-2.38)	

Contraceptives 1st trimester **N=324**	Yes	5 (1.8)	1 (2.0)
	No	269 (98.8)	49 (98.0)
	X^2 (df), P value OR (95% CI)	0.01 (1) 0.933 1.1 (0.13-9.6)	
Illness pre-gestation **N=324**	Yes	71 (28.7 0)	19 (38.0)
	No	203 (82.2)	31 (62.0)
	X^2 (df), P value OR (95% CI)	3.08 (1) 0.082 1.75 (0.93-3.3)	
Illness 1st trimester **N=324**	Yes	103 (41.7)	21 (42.0)
	No	171 (69.2)	29 (58.)
	X^2 (df), P value OR (95% CI)	0.35 (1) 0.56 1.2 (0.65-2.2)	
Common cold/flu pre-gestation **N=324**	Yes	53 (21.5)	13 (26.0)
	No	221 (89.5)	37 (74.0)
	X^2 (df), P value OR (95% CI)	1.16 (1) 0.285 1.47 (0.73-2.95)	
Common cold/flu 1st trimester **N=324**	Yes	57 (23.1)	15 (30.0)
	No	217 (87.9)	35 (70.0)
	X^2 (df), P value OR (95% CI)	2.07 (1) 0.15 1.66 (0.85-3.25)	
Fever pre-gestation **N=324**	Yes	28 (11.3)	6 (12.0)
	No	246 (99.6)	44 (88.0)
	X^2 (df), P value OR (95% CI)	0.14 (1) 0.71 1.2 (0.47-3.06)	
Fever 1st trimester **N=324**	Yes	39 (15.8)	7 (14.0)
	No	235 (95.1)	43 (86.0)
	X^2 (df), P value OR (95% CI)	0.00 (1) 0.965 0.98 (0.41-2.34)	
Urinary tract infection pre-gestation **N=322**	Yes	10 (4.1)	2 (4.0)
	No	262 (106.9)	48 (96.0)
	X^2 (df), P value OR (95% CI)	0.01 (1) 0.91 1.05 (0.22-4.93)	
Urinary tract infection 1st trimester **N=322**	Yes	10 (4.1)	0
	No	262 (106.9)	50 (100.0)
	X^2 (df), P value OR (95% CI)	1.90 (1) 0.17	
High blood pressure pre-	Yes	4 (1.6)	2 (4.0)

gestation **N=324**	No	270 (109.3)	48 (96.0)
	X^2 (df), P value OR (95% CI)	1.50 (1) 0.22 2.81 (0.5-15.78)	
High blood pressure 1st trimester **N=324**	Yes	4 (1.6)	4 (8.0)
	No	270 (109.3)	46 (92.0)
	X^2 (df), P value OR (95% CI)	7.51 (1) 0.01** 5.87 (1.42-24.3)	
Diabetes pre-gestation **N=324**	Yes	0 (0.0)	0 (0.0)
	No	274 (110.9)	50 (100.0)
	X^2 (df), P value OR (95% CI)	a	
Diabetes 1st trimester **N=324**	Yes	6 (2.4)	2 (4.0)
	No	268 (108.5)	48 (96.0)
	X^2 (df), P value OR (95% CI)	0.58 (1) 0.45 1.65 (0.48-5.62)	
Asthma pre-gestation **N=322**	Yes	4 (1.6)	2 (4.0)
	No	268 (109.4)	48 (96.0)
	X^2 (df), P value OR (95% CI)	1.48 (1) 0.22 2.19 (0.69-7)	
Asthma 1st trimester **N=322**	Yes	4 (1.6)	2 (4.0)
	No	268 (109.4)	48 (96.0)
	X^2 (df), P value OR (95% CI)	1.48 (1) 0.22 2.19 (0.69-7)	
Convulsions pre-gestation **N=324**	Yes	0 (0.0)	0 (0.0)
	No	274 (110.9)	50 (100.0)
	X^2 (df), P value OR (95% CI)	a	
Convulsions 1st trimester **N=324**	Yes	0 (0.0)	0 (0.0)
	No	274 (110.9)	50 (100.0)
	X^2 (df), P value OR (95% CI)	a	
Vaginal bleeding **N=322**	Yes	18 (7.4)	2 (4.0)
	No	254 (103.7)	48 (96.0)
	X^2 (df), P value OR (95% CI)	0.50 (1) 0.498 0.63 (0.16-2.4)	
Maternal supplement use			
Folic acid pre-gestation	Yes	24 (9.7)	8 (16.0)

N=324	No	250 (101.2)	42 (84.0)
	X^2 (df), P value OR (95% CI)	2.49 (1) 0.11 1.74 (0.9-3.37)	
Folic acid 1st trimester **N=326**	Yes	161 (65.2)	31 (61.8)
	No	114 (46.2)	20 (39.2)
	X^2 (df), P value OR (95% CI)	0.09 (1) 0.766 1.08 (0.65-1.81)	
Multivitamins pre-gestation **N=326**	Yes	16 (6.5)	4 (7.8)
	No	259 (104.9)	47 (92.2)
	X^2 (df), P value OR (95% CI)	0.31 (1) 0.572 1.3 (0.52-3.25)	
Multivitamins 1st trimester **N=324**	Yes	54 (21.9)	12 (24.0)
	No	220 (89.1)	38 (76.0)
	X^2 (df), P value OR (95% CI)	0.48 (1) 0.484 1.23 (0.68-2.23)	
Iron pre-gestation **N=324**	Yes	18 (7.3)	6 (12)
	No	256 (103.6)	44 (88.0)
	X^2 (df), P value OR (95% CI)	1.82 (1) 0.18 1.7 (0.81-3.59)	
Iron 1st trimester **N=324**	Yes	82 (33.2)	20 (40.0)
	No	192 (77.7)	30 (60.0)
	X^2 (df), P value OR (95% CI)	1.99 (1) 0.157 1.45 (0.87-2.43)	
Calcium 1st trimester **N=324**	Yes	15 (6.1)	1 (2.0)
	No	259 (104.9)	49 (98.0)
	X^2 (df), P value OR (95% CI)	1.09 (1) 0.30 0.39 (0.06-2.67)	
Smoking			
Maternal smoking **N=322**	Yes	10 (3.7)	2 (4.0)
	No	262 (96.3)	48 (96.0)
	X^2 (df), P value OR (95% CI)	0.01 (1) 0.91 1.08 (0.3-3.92)	
Paternal smoking **N=322**	Yes	94 (34.6)	20 (40.0)
	No	178 (65.4)	30 (60.0)
	X^2 (df), P value OR (95% CI)	0.55 (1) 0.458 1.22 (0.72-2.04)	
Paternal tobacco	Yes	74 (27.2)	12 (24.0)

N=322	No	198 (72.8)	38 (76.0)
	X^2 (df), P value OR (95% CI)	0.22 (1) 0.64 0.87 (0.48-1.58)	
Paternal waterpipe **N=324**	Yes	31 (11.4)	13 (25.5)
	No	242(88.6)	38 (74.5)
	X^2 (df), P value OR (95% CI)	7.32 (1) 0.01** 2.67 (1.28-5.56)	
Maternal passive smoking **N=322**	Yes	54 (19.9)	12 (24.0)
	No	218 (80.2)	38 (76.0)
	X^2 (df), P value OR (95% CI)	0.45 (1) 0.50 1.22 (0.68-2.21)	
Maternal stress			
Family problems **N=322**	Yes	101 (37.1)	25 (50)
	No	171 (62.9)	25 (50.0)
	X^2 (df), P value OR (95% CI)	2.94 (1) 0.088 1.69 (0.92-3.1)	
Mother complains of being under stress **N=322**	Yes	117 (43.0)	29 (58.0)
	No	155 (57.0)	21 (42.0)
	X^2 (df), P value OR (95% CI)	3.83 (1) 0.053 1.83 (0.99-3.37)	
Depression pre-gestation **N=324**	Yes	5 (1.8)	1 (2.0)
	No	269 (98.2)	49 (98.0)
	X^2 (df), P value OR (95% CI)	0.01 (1) 0.932 1.08 (0.18-6.59)	
Depression 1st trimester **N=324**	Yes	13 (4.7)	1 (2.0)
	No	261 (95.3)	49 (98.0)
	X^2 (df), P value OR (95% CI)	0.77 (1) 0.38 0.45 (0.07-3.04)	
Severe morning sickness **N=322**	Yes	32 (11.8)	8 (16.0)
	No	240 (88.2)	42 (84.0)
	X^2 (df), P value OR (95% CI)	0.70 (1) 0.40 1.34 (0.68-2.65)	
Threatened abortion **N=322**	Yes	18 (6.6)	2 (4.0)
	No	254 (93.4)	48 (96.0)
	X^2 (df), P value OR (95% CI)	0.50 (1) 0.498 0.63 (0.16-2.4)	
Abdominal pain pre-	Yes	10 (3.7)	2 (4.0)

gestation **N=322**	No	262 (96.3)	48 (96.0)
	X^2 (df), P value OR (95% CI)	0.01 (1) 0.911 1.08 (0.3-3.92)	
Abdominal pain 1st trimester **N=322**	Yes	27 (9.93)	9 (18.0)
	No	245 (90.1)	41 (82.0)
	X^2 (df), P value OR (95% CI)	2.77 (1) 0.10 1.74 (0.93-3.28)	
Maternal domestic environmental exposure			
Exposure to chemicals pre-gestation **N=466**	Yes	86 (31.6)	16 (8.25)
	No	186 (68.4)	178 (91.8)
	X^2 (df), P value OR (95% CI)	36.17 (1) <0.001** 0.19 (0.11-0.34)	
Exposure to chemicals 1st trimester **N=318**	Yes	84 (31.1)	14 (29.1)
	No	186 (68.9)	34 (70.8)
	X^2 (df), P value OR (95% CI)	0.07 (1) 0.80 0.92 (0.52-1.64)	
Exposure to solvents pre-gestation **N=322**	Yes	32 (11.8)	10 (20.0)
	No	240 (8.2)	40 (80)
	X^2 (df), P value OR (95% CI)	2.53 (1) 0.117 1.67 (0.9-3.07)	
Exposure to solvents 1st trimester **N=322**	Yes	24 (8.8)	8 (16.0)
	No	248 (91.2)	42 (84.0)
	X^2 (df), P value OR (95% CI)	2.43 (1) 0.12 1.73 (0.89-3.35)	
Exposure to pesticides pre-gestation **N=322**	Yes	44 (16.2)	4 (8.0)
	No	228 (83.8)	46 (92.0)
	X^2 (df), P value OR (95% CI)	2.23 (1) 0.14 0.5 (0.19-1.32)	
Exposure to pesticides 1st trimester **N=322**	Yes	51 (18.8)	5 (10.0)
	No	221 (81.3)	45 (90.0)
	X^2 (df), P value OR (95% CI)	2.25 (1) 0.13 0.53 (0.22-1.27)	
Exposure to incense pre-gestation **N=322**	Yes	103 (37.9)	13 (26.0)
	No	169 (62.1)	37 (74.0)
	X^2 (df), P value OR (95% CI)	2.58 (1) 0.11 0.62 (0.35-1.13)	
Exposure to incense 1st	Yes	102 (40.2)	14 (43.8)

trimester **N=286**	No	152 (59.8)	18 (56.3)
	X^2 (df), P value OR (95% CI)	0.15 (1) 0.70 1.14 (0.59-2.2)	
Maternal exposure to X-ray 1st trimester **N=326**	Yes	10 (3.6)	2 (3.9)
	No	265 (96.4)	49 (96.1)
	X^2 (df), P value OR (95% CI)	0.01 (1) 0.92 1.07 (0.29-3.88)	
Type of maternal drinking water 0.36 (1) 0.95 **N=318**	Tap	67 (25)	13 (26)
	Bottled	180 (67.2)	32 (64)
	P value OR (95% CI)	0.06 (1) 0.81 0.92 (0.45-1.9)	
	Well	16(6)	4 (8)
	P value OR (95% CI)	0.686 1.29 (0.37-4.48)	
	Zamzam	5 (1.9)	1 (2)
	P value OR (95% CI)	0.979 1.03 (0.11-9.56)	
Consanguinity **N=312**	Yes	149 (57.1)	27 (52.9)
	No	112 (42.9)	24 (47.1)
	X^2 (df), P value OR (95% CI)	0.30 (1) 0.58 0.87 (0.53-1.44)	
Family history **N=318**	Yes	112 (41.6)	22 (44.9)
	No	157 (58.4)	27 (55.1)
	X^2 (df), P value OR (95% CI)	0.18 (1) 0.67 1.12 (0.67-1.88)	
Maternal VAX_7078160		G* (%)	A (%)
Maternal medication use and illness			
Antibiotic pre-gestation **N=324**	Yes	37 (13.5)	7 (14.0)
	No	237 (86.5)	43 (86.0)
	X^2 (df), P value OR (95% CI)	0.01 (1) 0.925 1.04 (0.44-2.49)	
Antibiotic at 1st trimester **N=324**	Yes	46 (16.8)	4 (8.0)
	No	228 (83.2)	46 (92.0)
	X^2 (df), P value OR (95% CI)	2.50 (1) 0.11 0.43 (0.15-1.26)	
Antipyretic medication pre-gestation	Yes	19 (6.9)	3 (5.9)
	No	256 (93.1)	48 (94.1)

N=326	X^2 (df), P value OR (95% CI)	0.07 (1) 0.79 0.85 (0.24-2.99)	
Antipyretic medication 1st trimester **N=328**	Yes	27 (9.9)	5 (9.1)
	No	246 (90.1)	50 (90.9)
	X^2 (df), P value OR (95% CI)	0.03 (1) 0.855 0.91 (0.33-2.48)	
Anti-emetic medication pre-gestation **N=324**	Yes	2 (0.7)	0.(0.0)
	No	272 (99.3)	50 (100.0)
	X^2 (df), P value OR (95% CI)	0.37 (1) 0.54	
Anti-emetic medication 1st trimester **N=324**	Yes	42 (15.3)	6 (12)
	No	232 (84.7)	44 (88.0)
	X^2 (df), P value OR (95% CI)	0.37 (1) 0.544 0.75 (0.3-1.88)	
Contraceptives pre-gestation **N=320**	Yes	23 (8.5)	3 (6.0)
	No	247 (91.5)	47 (94.0)
	X^2 (df), P value OR (95% CI)	0.36 (1) 0.552 0.69 (0.2-2.38)	
Contraceptives 1st trimester **N=324**	Yes	5 (1.8)	1 (2.0)
	No	269 (98.8)	49 (98.0)
	X^2 (df), P value OR (95% CI)	0.01 (1) 0.933 1.1 (0.13-9.6)	
Illness pre-gestation **N=324**	Yes	71 (28.7 0)	19 (38.0)
	No	203 (82.2)	31 (62.0)
	X^2 (df), P value OR (95% CI)	3.08 (1) 0.082 1.75 (0.93-3.3)	
Illness 1st trimester **N=324**	Yes	103 (41.7)	21 (42.0)
	No	171 (69.2)	29 (58.)
	X^2 (df), P value OR (95% CI)	0.35 (1) 0.56 1.2 (0.65-2.2)	
Common cold/flu pre-gestation **N=324**	Yes	53 (21.5)	13 (26.0)
	No	221 (89.5)	37 (74.0)
	X^2 (df), P value OR (95% CI)	1.16 (1) 0.285 1.47 (0.73-2.95)	
Common cold/flu 1st trimester	Yes	57 (23.1)	15 (30.0)
	No	217 (87.9)	35 (70.0)

N=324	X^2 (df), P value OR (95% CI)	2.07 (1) 0.15 1.66 (0.85-3.25)	
Fever pre-gestation **N=324**	Yes	28 (11.3)	6 (12.0)
	No	246 (99.6)	44 (88.0)
	X^2 (df), P value OR (95% CI)	0.14 (1) 0.71 1.2 (0.47-3.06)	
Fever 1st trimester **N=324**	Yes	39 (15.8)	7 (14.0)
	No	235 (95.1)	43 (86.0)
	X^2 (df), P value OR (95% CI)	0.00 (1) 0.965 0.98 (0.41-2.34)	
Urinary tract infection pre-gestation **N=322**	Yes	10 (4.1)	2 (4.0)
	No	262 (106.9)	48 (96.0)
	X^2 (df), P value OR (95% CI)	0.01 (1) 0.91 1.05 (0.22-4.93)	
Urinary tract infection 1st trimester **N=322**	Yes	10 (4.1)	0
	No	262 (106.9)	50 (100.0)
	X^2 (df), P value OR (95% CI)	1.90 (1) 0.17	
High blood pressure pre-gestation **N=324**	Yes	4 (1.6)	2 (4.0)
	No	270 (109.3)	48 (96.0)
	X^2 (df), P value OR (95% CI)	1.50 (1) 0.22 2.81 (0.5-15.78)	
High blood pressure 1st trimester **N=324**	Yes	4 (1.6)	4 (8.0)
	No	270 (109.3)	46 (92.0)
	X^2 (df), P value OR (95% CI)	7.51 (1) 0.01 5.87 (1.42-24.3)	
Diabetes pre-gestation **N=324**	Yes	0 (0.0)	0 (0.0)
	No	274 (110.9)	50 (100.0)
	X^2 (df), P value OR (95% CI)	a	
Diabetes 1st trimester **N=324**	Yes	6 (2.4)	2 (4.0)
	No	268 (108.5)	48 (96.0)
	X^2 (df), P value OR (95% CI)	0.58 (1) 0.45 1.65 (0.48-5.62)	
Asthma pre-gestation **N=322**	Yes	4 (1.6)	2 (4.0)
	No	268 (109.4)	48 (96.0)

	X^2 (df), P value OR (95% CI)	1.48 (1) 0.22 2.19 (0.69-7)	
Asthma 1st trimester **N=322**	Yes	4 (1.6)	2 (4.0)
	No	268 (109.4)	48 (96.0)
	X^2 (df), P value OR (95% CI)	1.48 (1) 0.22 2.19 (0.69-7)	
Convulsions pre-gestation **N=324**	Yes	0 (0.0)	0 (0.0)
	No	274 (110.9)	50 (100.0)
	X^2 (df), P value OR (95% CI)	a	
Convulsions 1st trimester **N=324**	Yes	0 (0.0)	0 (0.0)
	No	274 (110.9)	50 (100.0)
	X^2 (df), P value OR (95% CI)	a	
Vaginal bleeding **N=322**	Yes	18 (7.4)	2 (4.0)
	No	254 (103.7)	48 (96.0)
	X^2 (df), P value OR (95% CI)	0.50 (1) 0.498 0.63 (0.16-2.4)	
Maternal supplement use			
Folic acid pre-gestation **N=324**	Yes	24 (9.7)	8 (16.0)
	No	250 (101.2)	42 (84.0)
	X^2 (df), P value OR (95% CI)	2.49 (1) 0.11 1.74 (0.9-3.37)	
Folic acid 1st trimester **N=326**	Yes	161 (65.2)	31 (61.8)
	No	114 (46.2)	20 (39.2)
	X^2 (df), P value OR (95% CI)	0.09 (1) 0.766 1.08 (0.65-1.81)	
Multivitamins pre-gestation **N=326**	Yes	16 (6.5)	4 (7.8)
	No	259 (104.9)	47 (92.2)
	X^2 (df), P value OR (95% CI)	0.31 (1) 0.572 1.3 (0.52-3.25)	
Multivitamins 1st trimester **N=324**	Yes	54 (21.9)	12 (24.0)
	No	220 (89.1)	38 (76.0)
	X^2 (df), P value OR (95% CI)	0.48 (1) 0.484 1.23 (0.68-2.23)	
Iron pre-gestation **N=324**	Yes	18 (7.3)	6 (12)
	No	256 (103.6)	44 (88.0)

	X^2 (df), P value OR (95% CI)	1.82 (1) 0.18 1.7 (0.81-3.59)	
Iron 1st trimester **N=324**	Yes	82 (33.2)	20 (40.0)
	No	192 (77.7)	30 (60.0)
	X^2 (df), P value OR (95% CI)	1.99 (1) 0.157 1.45 (0.87-2.43)	
Calcium 1st trimester **N=324**	Yes	15 (6.1)	1 (2.0)
	No	259 (104.9)	49 (98.0)
	X^2 (df), P value OR (95% CI)	1.09 (1) 0.30 0.39 (0.06-2.67)	
Smoking			
Maternal smoking **N=322**	Yes	10 (3.7)	2 (4.0)
	No	262 (96.3)	48 (96.0)
	X^2 (df), P value OR (95% CI)	0.01 (1) 0.91 1.08 (0.3-3.92)	
Paternal smoking **N=322**	Yes	94 (34.6)	20 (40.0)
	No	178 (65.4)	30 (60.0)
	X^2 (df), P value OR (95% CI)	0.55 (1) 0.458 1.22 (0.72-2.04)	
Paternal tobacco **N=322**	Yes	74 (27.2)	12 (24.0)
	No	198 (72.8)	38 (76.0)
	X^2 (df), P value OR (95% CI)	0.22 (1) 0.64 0.87 (0.48-1.58)	
Paternal waterpipe **N=324**	Yes	31 (11.4)	13 (25.5)
	No	242(88.6)	38 (74.5)
	X^2 (df), P value OR (95% CI)	7.32 (1) 0.01 2.67 (1.28-5.56)	
Maternal passive smoking **N=322**	Yes	54 (19.9)	12 (24.0)
	No	218 (80.2)	38 (76.0)
	X^2 (df), P value OR (95% CI)	0.45 (1) 0.50 1.22 (0.68-2.21)	
Maternal stress			
Family problems **N=322**	Yes	101 (37.1)	25 (50)
	No	171 (62.9)	25 (50.0)
	X^2 (df), P value OR (95% CI)	2.94 (1) 0.088 1.69 (0.92-3.1)	
Mother complains of	Yes	117 (43.0)	29 (58.0)

being under stress **N=322**	No	155 (57.0)	21 (42.0)
	X^2 (df), P value OR (95% CI)	3.83 (1) 0.053 1.83 (0.99-3.37)	
Depression pre-gestation **N=324**	Yes	5 (1.8)	1 (2.0)
	No	269 (98.2)	49 (98.0)
	X^2 (df), P value OR (95% CI)	0.01 (1) 0.932 1.08 (0.18-6.59)	
Depression 1st trimester **N=324**	Yes	13 (4.7)	1 (2.0)
	No	261 (95.3)	49 (98.0)
	X^2 (df), P value OR (95% CI)	0.77 (1) 0.38 0.45 (0.07-3.04)	
Severe morning sickness **N=322**	Yes	32 (11.8)	8 (16.0)
	No	240 (88.2)	42 (84.0)
	X^2 (df), P value OR (95% CI)	0.70 (1) 0.40 1.34 (0.68-2.65)	
Threatened abortion **N=322**	Yes	18 (6.6)	2 (4.0)
	No	254 (93.4)	48 (96.0)
	X^2 (df), P value OR (95% CI)	0.50 (1) 0.498 0.63 (0.16-2.4)	
Abdominal pain pre-gestation **N=322**	Yes	10 (3.7)	2 (4.0)
	No	262 (96.3)	48 (96.0)
	X^2 (df), P value OR (95% CI)	0.01 (1) 0.911 1.08 (0.3-3.92)	
Abdominal pain 1st trimester **N=322**	Yes	27 (9.93)	9 (18.0)
	No	245 (90.1)	41 (82.0)
	X^2 (df), P value OR (95% CI)	2.77 (1) 0.10 1.74 (0.93-3.28)	
Maternal domestic environmental exposure			
Exposure to chemicals pre-gestation **N=466**	Yes	86 (31.6)	16 (8.25)
	No	186 (68.4)	178 (91.8)
	X^2 (df), P value OR (95% CI)	36.17 (1) <0.001 0.19 (0.11-0.34)	
Exposure to chemicals 1st trimester **N=318**	Yes	84 (31.1)	14 (29.1)
	No	186 (68.9)	34 (70.8)
	X^2 (df), P value OR (95% CI)	0.07 (1) 0.80 0.92 (0.52-1.64)	
Exposure to solvents pre-	Yes	32 (11.8)	10 (20.0)

gestation **N=322**	No	240 (8.2)	40 (80)
	X^2 (df), P value OR (95% CI)	2.53 (1) 0.117 1.67 (0.9-3.07)	
Exposure to solvents 1st trimester **N=322**	Yes	24 (8.8)	8 (16.0)
	No	248 (91.2)	42 (84.0)
	X^2 (df), P value OR (95% CI)	2.43 (1) 0.12 1.73 (0.89-3.35)	
Exposure to pesticides pre-gestation **N=322**	Yes	44 (16.2)	4 (8.0)
	No	228 (83.8)	46 (92.0)
	X^2 (df), P value OR (95% CI)	2.23 (1) 0.14 0.5 (0.19-1.32)	
Exposure to pesticides 1st trimester **N=322**	Yes	51 (18.8)	5 (10.0)
	No	221 (81.3)	45 (90.0)
	X^2 (df), P value OR (95% CI)	2.25 (1) 0.13 0.53 (0.22-1.27)	
Exposure to incense pre-gestation **N=322**	Yes	103 (37.9)	13 (26.0)
	No	169 (62.1)	37 (74.0)
	X^2 (df), P value OR (95% CI)	2.58 (1) 0.11 0.62 (0.35-1.13)	
Exposure to incense 1st trimester **N=286**	Yes	102 (40.2)	14 (43.8)
	No	152 (59.8)	18 (56.3)
	X^2 (df), P value OR (95% CI)	0.15 (1) 0.70 1.14 (0.59-2.2)	
Maternal exposure to X-ray 1st trimester **N=326**	Yes	10 (3.6)	2 (3.9)
	No	265 (96.4)	49 (96.1)
	X^2 (df), P value OR (95% CI)	0.01 (1) 0.92 1.07 (0.29-3.88)	
Type of maternal drinking water 0.36 (1) 0.95 **N=318**	Tap	67 (25)	13 (26)
	Bottled	180 (67.2)	32 (64)
	P value OR (95% CI)	0.06 (1) 0.81 0.92 (0.45-1.9)	
	Well	16(6)	4 (8)
	P value OR (95% CI)	0.686 1.29 (0.37-4.48)	
	Zamzam	5 (1.9)	1 (2)
	P value OR (95% CI)	0.979 1.03 (0.11-9.56)	

Consanguinity **N=312**	Yes	149 (57.1)	27 (52.9)
	No	112 (42.9)	24 (47.1)
	X^2 (df), P value OR (95% CI)	0.30 (1) 0.58 0.87 (0.53-1.44)	
Family history **N=318**	Yes	112 (41.6)	22 (44.9)
	No	157 (58.4)	27 (55.1)
	X^2 (df), P value OR (95% CI)	0.18 (1) 0.67 1.12 (0.67-1.88)	

**The Chi-square statistic is significant at the 0.05 level

* Homozygous common allele genotype

[a] Not possible to analyse because the groups contain zero values

If one of the cells contains zero value, the OR and 95% CI are not calculated

A50: Comparison between cases and controls for maternal *VAX1* rs7078160 genotypes in relationship to different environmental factors including: maternal illness, medication, stress, supplements, paternal smoking and maternal domestic exposure.

Maternal VAX_7078160		**GG**** (%)		AA (%)		AG (%)	
Environmental factors		Study	Control	Study	Control	Study	Control
Antibiotic use pre-gestation **Study Group N=162 Control Group N=177**	Yes	18 (14.8)	9 (5.8)	3 (30.0)	0 (0.0)	1 (3.3)	0 (0.0)
	No	104 (85.2)	146 (94.2)	7 (70.0)	5 (100.0)	29 (96.7)	17 (100.0)
	X^2 (df), P value OR (95% CI)	6.21 (1), 0.013** 2.81 (1.21-6.5)		1.88 (1), 0.171		0.58 (1), 0.447	
Antibiotic use 1st trimester **Study Group N=162 Control Group N=177**	Yes	22 (18.0)	16 (10.3)	1 (10.0)	0 (0.0)	2 (6.7)	2 (11.8)
	No	100 (82.0)	139 (89.7)	9 (90.0)	5 (100.0)	28 (93.3)	15 (88.2)
	X^2 (df), P value	3.43 (1), 0.064		0.54 (1), 0.464		0.36 (1), 0.547	
Antipyretic medication pre-gestation **Study Group N=162 Control Group N=177**	Yes	8 (6.6)	16 (10.3)	0 (0.0)	0 (0.0)	3 (9.7)	2 (11.8)
	No	114 (93.4)	139 (89.7)	10 (100.0)	5 (100.0)	28 (90.3)	15 (88.2)
	X^2 (df), P value	1.22 (1), 0.269		a		0.051 (1), 0.218	
Antipyretic medication 1st trimester **Study Group N=161 Control Group N=174**	Yes	12 (10.0)	21 (13.7)	1 (10.0)	0 (0.0)	3 (9.7)	4 (25.0)
	No	108 (90.0)	132 (86.3)	9 (90.0)	5 (100.0)	28 (90.3)	12 (75.0)
	X^2 (df), P value	0.88 (1), 0.349		0.54 (1), 0.464		1.96 (1), 0.162	
Anti-emetic medication pre-gestation **Study Group N=162 Control Group N=177**	Yes	1 (0.8)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	No	121 (99.2)	155 (100.0)	10 (100.0)	5 (100.0)	30 (100.0)	17 (100.0)
	X^2 (df), P value	1.28 (1|), 0.259		a		a	
Anti-emetic medication 1st trimester	Yes	20 (16.4)	13 (8.4)	2 (20.0)	0 (0.0)	2 (6.7)	1 (5.9)
	No	102 (83.6)	142 (91.6)	8 (80.0)	5 (100.0)	28 (93.3)	16 (94.1)

Study Group N=162 Control Group N=177	X^2 (df), P value OR (95% CI)	4.17 (1), 0.041** 2.14 (1.02-4.5)		1.15 (1), 0.283		0.01 (1), 0.916	
Contraceptives pre-gestation **Study Group N=160 Control Group N=177**	Yes	10 (8.3)	14 (9.0)	0 (0.0)	0 (0.0)	3 (10.0)	0 (0.0)
	No	110 (91.7)	141 (91.0)	10 (100.0)	5 (100.0)	27 (90.0)	17 (100.0)
	X^2 (df), P value	1.82 (1), 0.839		a		0.178	
Contraceptives 1st trimester **Study Group N=162 Control Group N=177**	Yes	2 (1.6)	5 (3.2)	0 (0.0)	0 (0.0)	1 (3.3)	0 (0.0)
	No	120 (98.4)	150 (96.8)	10 (100.0)	5 (100.0)	29 (96.7)	17 (100.0)
	X^2 (df), P value	0.7 (1), 0.404		a		0.58 (1), 0.447	
Illness pre-gestation **Study Group N=162 Control Group N=176**	Yes	30 (24.6)	13 (8.4)	4 (40.0)	1 (20.0)	11 (36.7)	5 (29.4)
	No	92 (75.4)	141 (91.6)	6 (60.0)	4 (80.0)	19 (63.3)	12 (70.6)
	X^2 (df), P value OR (95% CI)	13.5 (1), <0.001** 3.54 (1.75-7.13)		0.6 (1), 0.439		0.25 (1), 0.614	
Illness 1st trimester **Study Group N=162 Control Group N=175**	Yes	46 (37.7)	39 (25.5)	5 (50.0)	2 (40.0)	11 (36.7)	6 (35.3)
	No	76 (62.3)	114 (74.5)	5 (50.0)	3 (60.0)	19 (63.3)	11 (64.7)
	X^2 (df), P value OR (95% CI)	43.74 (1), 0.029** 1.77 (1.06-2.96)		0.13 (1), 0.714		0.009 (1), 0.925	
Common cold/flu pre-gestation **Study Group N=162 Control Group N=175**	Yes	22 (18.0)	6 (3.9)	2 (20.06)	0 (0.0)	9 (30.0)	1 (5.9)
	No	100 (82)	147 (96.1)	8 (80.0)	5 (100.0)	21 (70.0)	16 (94.1)
	X^2 (df), P value OR (95% CI)	14.78 (1), <0.001** 5.39 (2.11-13.77)		1.15 (1), 0.283		3.77 (1), 0.052	
Common cold/flu 1st trimester **Study Group N=162 Control Group N=175**	Yes	25 (20.5)	21 (13.7)	4 (40.0)	0 (0.0)	7 (23.3)	3 (17.6)
	No	97 (79.5)	132 (86.3)	6 (60.0)	5 (100.0)	23 (76.7)	14 (82.4)
	X^2 (df), P value	2.23 (1), 0.135		2.73 (1), 0.099		0.21 (1), 0.647	
Fever pre-gestation **Study Group N=162 Control Group N=175**	Yes	12 (9.8)	7 (4.6)	1 (10.0)	1 (20.0)	4 (13.3)	0 (0.0)
	No	110 (90.2)	146 (95.4)	9 (90.0)	4 (80.0)	26 (86.7)	17 (100.0)
	X^2 (df), P value	2.92 (1), 0.087		0.29 (1), 0.591		2.48 (1), 0.115	
Fever 1st trimester **Study Group N=162 Control Group N=175**	Yes	18 (14.8)	12 (7.8)	2 (20.0)	1 (20.0)	3 (10.0)	2 (11.8)
	No	104 (85.2)	141 (92.2)	8 (80.0)	4 (80.0)	27 (90.0)	15 (88.2)
	X^2 (df), P value	3.34 (1), 0.068		0.000 (1), 1		0.04 (1), 0.85	

Urinary tract infection pre-gestation **Study Group N=161 Control Group N=175**	Yes	5 (4.1)	3 (2.0)	1 (10.0)	0 (0.0)	0 (0.0)	1 (5.9)
	No	116 (95.9)	150 (98.0)	9 (90.0)	5 (100.0)	30 (100.0)	16 (94.1)
	X^2 (df), P value	1.12 (1), 0.289		0.54 (1), 0.464		1.8 (1), 0.179	
Urinary tract infection 1st trimester **Study Group N=161 Control Group N=175**	Yes	5 (4.1)	6 (3.9)	0 (0.0)	0 (0.0)	0 (0.0)	1 (5.9)
	No	116 (95.9)	147 (96.1)	10 (100)	5 (100.0)	30 (100.0)	16 (94.1)
	X^2 (df), P value	0.008 (1), 0.93		a		1.8 (1), 0.179	
High blood pressure pre-gestation **Study Group N=162 Control Group N=175**	Yes	2 (1.6)	1 (0.7)	1 (10.0)	0 (0.0)	0 (0.0)	0 (0.0)
	No	120 (98.4)	152 (99.3)	9 (90.0)	5 (100.0)	30 (100.0)	17 (100.0)
	X^2 (df), P value	0.61 (1), 0.434		0.54 (1), 0.646		a	
High blood pressure 1st trimester **Study Group N=162 Control Group N=175**	Yes	2 (1.6)	2 (1.3)	2 (20.0)	0 (0.0)	0 (0.0)	0 (0.0)
	No	120 (98.4)	151 (98.7)	8 (80.0)	5 (100.0)	30 (100.0)	17 (100.0)
	X^2 (df), P value	0.05 (1), 0.819		1.15 (1), 0.283		a	
Diabetes pre-gestation **Study Group N=162 Control Group N=175**	Yes	0 (0.0)	1 (0.7)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	No	122 (100.0)	152 (99.3)	10 (100.0)	5 (100.0)	30 (100.0)	17 (100.0)
	X^2 (df), P value	0.8 (1), 0.371		a		a	
Diabetes 1st trimester **Study Group N=162 Control Group N=175**	Yes	3 (2.5)	3 (2.0)	1 (10.0)	0 (0.0)	0 (0.0)	0 (0.0)
	No	119 (97.5)	150 (98.0)	9 (90.0)	5 (100.0)	30 (100.0)	17 (100.0)
	X^2 (df), P value	0.779		0.464		a	
Asthma pre-gestation **Study Group N=161 Control Group N=175**	Yes	1 (0.8)	0 (0.0)	0 (0.0)	0 (0.0)	2 (6.7)	1 (5.9)
	No	120 (99.2)	153 (100.0)	10 (100.0)	5 (100.0)	28 (93.3)	16 (94.1)
	X^2 (df), P value	1.27 (1), 0.26		a		0.01 (1), 0.916	
Asthma 1st trimester **Study Group N=161 Control Group N=175**	Yes	1 (0.8)	3 (2.0)	0 (0.0)	0 (0.0)	2 (6.7)	1 (5.9)
	No	120 (99.2)	150 (98.0)	10 (100.0)	5 (100.0)	28 (93.3)	16 (94.1)
	X^2 (df), P value	0.6 (1), 0.437		a		0.92 (1),0.916	

Convulsions pre-gestation **Study Group N=162 Control Group N=175**	Yes	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	No	122 (100.0)	153 (100.0)	10 (100.0)	5 (100.0)	30 (100.0)	17 (100.0)
	X^2 (df), P value	a		a		a	
Convulsions 1st trimester **Study Group N=162 Control Group N=175**	Yes	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
	No	122 (100.0)	153 (100.0)	10 (100.0)	5 (100.0)	30 (100.0)	17 (100.0)
	X^2 (df), P value	a		a		a	
Vaginal bleeding **Study Group N=161 Control Group N=175**	Yes	8 (6.6)	5 (3.3)	0 (0.0)	0 (0.0)	2 (6.7)	0 (0.0)
	No	113 (93.4)	148 (96.7)	10 (100.0)	5 (100.0)	28 (93.3)	17 (100.0)
	X^2 (df), P value	1.67 (1), 0.196		a		1.18 (1), 0.277	
Maternal exposure to x-ray in the 1st trimester **Study Group N=163 Control Group N=177**	Yes	4 (3.3)	2 (1.3)	0 (0.0)	0 (0.0)	2 (6.5)	0 (0.0)
	No	118 (96.7)	153 (98.7)	10 (100.0)	5 (100.0)	29 (93.5)	17 (100.0)
	X^2 (df), P value	1.27 (1), 0.259		a		1.14 (1), 0.285	
Maternal supplement use							
Folic acid pre-gestation **Study Group N=162 Control Group N=204**	Yes	10 (8.2)	41 (9.0)	2 (20.0)	1 (20.0)	4 (12.9)	1 (5.9)
	No	112 (91.8)	141 (91.0)	8 (80.0)	4 (80.0)	26(87.1)	16 (94.1)
	X^2 (df), P value	0.06 (1), 0.806		0.000 (1), 1		0.58 (1), 0.446	
Folic acid 1st trimester **Study Group N=163 Control Group N=177**	Yes	72 (59.0)	111 (71.6)	7 (70.0)	4 (80.0)	17 (54.8)	11 (64.7)
	No	50 (41.0)	44 (28.4)	3 (30.0)	1 (20.0)	14 (45.2)	6 (35.3)
	X^2 (df), P value OR (95% CI)	4.8 (1), 0.028** 0.57 (0.35-0.94)		0.17 (1), 0.68		0.44 (1), 0.507	
Multivitamins pre-gestation **Study Group N=163 Control Group N=177**	Yes	6 (4.9)	3 (1.9)	0 (0.0)	1 (20.0)	4 (12.9)	0 (0.0)
	No	116 (95.1)	152 (98.1)	10 (100.0)	4 (80)	27 (87.1)	17 (100.0)
	X^2 (df), P value	1.93 (1), 0.165		2.15 (1), 0.143		2.39 (1), 0.122	
Multivitamins 1st trimester **Study Group N=162 Control Group N=177**	Yes	24 (19.7)	26 (16.8)	3 (30.0)	3 (60.0)	6 (20.0)	3 (17.6)
	No	98 (80.3)	129 (83.2)	7 (70.0)	2 (40.0)	24 (80.0)	14 (82.4)
	X^2 (df), P value	0.39 (1), 0.534		1.25 (1), 0.264		0.4 (1), 0.844	

Iron pre-gestation **Study Group N=162 Control Group N=177**	Yes	6 (4.9)	8 (5.2)	0 (0.0)	0 (0.0)	6 (20.0)	2 (11.8)
	No	116 (95.1)	147 (94.8)	10 (100.0)	5 (100.0)	24 (80.0)	15 (88.2)
	X^2 (df), P value	0.008 (1), 0.927		a		0.52 (1), 0.47	
Iron 1st trimester **Study Group N=162 Control Group N=177**	Yes	36 (29.5)	53 (34.2)	5 (50.0)	3 (60.0)	10 (33.3)	4 (23.5)
	No	86 (70.5)	102 (65.8)	5 (50.0)	2 (40.0)	20 (66.7)	13 (76.5)
	X^2 (df), P value	0.69 (1), 0.407		0.13 (1), 0.714		0.5 (1), 0.48	
Calcium 1st trimester **Study Group N=162 Control Group N=177**	Yes	7 (5.7)	16 (10.3)	0 (0.0)	1 (20.0)	1 (3.3)	3 (17.6)
	No	115 (94.3)	139 (89.7)	10 (100.0)	4 (80.0)	29 (96.7)	14 (82.4)
	X^2 (df), P value	1.89 (1), 0.17		2.14 (1), 0.143		2.86 (1), 0.091	
Smoking							
Maternal smoking **Study Group N=161 Control Group N=177**	Yes	4 (3.3)	5 (3.2)	0 (0.0)	2 (40.0)	2 (6.7)	1 (5.9)
	No	117 (96.7)	150 (96.8)	10 (100.0)	3 (60.0)	28 (93.3)	16 (94.1)
	X^2 (df), P value	0.001 (1), 0.97		4.62 (1), 0.032**		00.01 (1), 0.916	
Paternal smoking **Study Group N=161 Control Group N=177**	Yes	43 (35.5)	56 (36.1)	6 (60.0)	3 (60.0)	8 (26.7)	3 (17.6)
	No	78 (64.5)	99 (63.9)	4 (40.0)	2 (40.0)	22 (73.3)	14 (82.4)
	X^2 (df), P value	0.01 (1), 0.919		0.000 (1), 1		0.49 (1), 0.483	
Paternal tobacco **Study Group N=161 Control Group N=177**	Yes	35 (28.9)	46 (29.7)	4 (40.0)	3 (60.0)	4 (13.3)	3 (17.6)
	No	86 (71.7)	109 (70.3)	6 (60.0)	2 (40.0)	26 (86.7)	14 (82.4)
	X^2 (df), P value	0.02 (1), 0.892		0.54 (1), 0.464		0.16 (1), 0.69	
Waterpipe smoking **Study Group N=161 Control Group N=177**	Yes	13 (10.7)	10 (6.5)	4 (40)	0	5 (16.7)	0
	No	108 (89.3)	145 (93.5)	6 (60)	5 (100)	25 (83.3)	17 (100)
	X^2 (df), P value	1.64 (1), 0.2		2.73 (1), 0.099		3.17 (1), 0.075	
Paternal Jorak **Study Group N=161 Control Group N=177**	Yes	9 (7.4)	2 (1.3)	1 (10.0)	0 (0.0)	3 (10.0)	0 (0.0)
	No	112 (92.6)	153 (98.7)	9 (90.0)	5 (100.0)	27 (90.0)	17 (100.0)
	X^2 (df), P value OR (95% CI)	6.7 (1), 0.010** 6.15 (1.3-29)		0.54 (1), 0.464		1.82 (1), 0.178	
Paternal Moasel **Study Group N=161 Control Group N=177**	Yes	6 (5.0)	9 (5.8)	3 (30.0)	0 (0.0)	3 (10.0)	0 (0.0)
	No	115 (95.0)	146 (94.2)	7 (70.0)	5 (100.0)	27 (90.0)	17 (100.0)
	X^2 (df), P value	01 (1), 0.758		1.88 (1), 0.171		1.82 (1), 0.178	
Maternal passive	Yes	25 (20.7)	27 (17.4)	4 (40.0)	2 (40.0)	4 (13.3)	3 (17.6)

smoking **Study Group N=161** **Control Group N=177**	No	96 (79.3)	128 (82.6)	6 (60.0)	3 (60.0)	26 (86.7)	14 (82.4)
	X^2 (df), P value	0.35 (2), 0.494		0.83 (2), 1		2.24 (2), 0.69	
Maternal stress							
Family problems **Study Group N=161** **Control Group N=177**	Yes	43 (35.5)	36 (23.2)	5 (50.0)	2 (40.0)	15 (50.0)	5 (29.4)
	No	78 (64.5)	119 (76.8)	5 (50.0)	3 (60.0)	15 (50.0)	12 (70.6)
	X^2 (df), P value OR (95% CI)	5.04 (1), 0.025** 1.82 (1.08-3.09)		0.13 (1), 0.714		1.88 (1), 0.17	
Mother complains of being under stress **Study Group N=161** **Control Group N=177**	Yes	50 (41.3)	45 (29.0)	6 (60.0)	3 (60.0)	17 (56.7)	6 (35.3)
	No	71 (58.7)	110 (71.0)	4 (40.0)	2 (40.0)	13 (43.3)	11 (64.7)
	X^2 (df), P value OR (95% CI)	4.55 (1), 0.033** 1.7 (1.04-2.84)		0.000 (1), 1		1.98 (1), 0.159	
Depression pre-gestation **Study Group N=162** **Control Group N=175**	Yes	2 (1.6)	2 (1.3)	0 (0.0)	0 (0.0)	1 (3.3)	0 (0.0)
	No	120 (98.4)	151 (98.7)	10 (100.0)	5 (100)	29 (96.7)	17 (100.0)
	X^2 (df), P value	0.05 (1), 0.819		a		0.58 (1), 0.447	
Depression 1st trimester **Study Group N=162** **Control Group N=175**	Yes	6 (4.9)	3 (2.0)	0 (0.0)	0(0.0)	1 (3.3)	0 (0.0)
	No	116 (95.1)	150 (98.0)	10 (100.0)	5 (100.0)	29 (96.7)	17 (100.0)
	X^2 (df), P value	1.88 (1), 0.171		a		0.58 (1), 0.447	
Severe morning sickness **Study Group N=161** **Control Group N=175**	Yes	13 (10.7)	10 (6.5)	1 (10)	1 (20)	6 (6.5)	1 (5.9)
	No	108 (89.3)	143 (93.5)	9 (90)	4 (80)	24 (93.5)	16 (94.1)
	X^2 (df), P value	1.56 (1), 0.212		0.29 (1), 0.591		1.71 (1), 0.191	
Threatened abortion **Study Group N=161** **Control Group N=175**	Yes	8 (6.6)	4 (2.6)	0 (0.0)	0 (0.0)	2 (6.7)	3 (17.6)
	No	113 (93.4)	149 (97.4)	10 (100.0)	5 (100.0)	28 (93.3)	14 (82.4)
	X^2 (df), P value	2.58 (1, 0.108		a		1.38 (1), 0.241	
Abdominal pain pre-gestation **Study Group N=161** **Control Group N=175**	Yes	4 (3.3)	4 (2.6)	0 (0.0)	1 (20.0)	2 (6.7)	0 (0.0)
	No	117 (96.7)	149 (97.4)	10 (100.0)	4 (80.0)	28 (93.3)	17 (100.0)
	X^2 (df), P value	0.11 (1), 0.736		2.14 (1), 0.143		1.18 (1), 0.277	
Abdominal pain 1st trimester **Study Group**	Yes	11 (9.1)	7 (4.6)	2 (20.0)	1 (20.0)	5 (16.7)	3 (17.6)
	No	110 (90.9)	146 (95.4)	8 (80.0)	4 (80.0)	25 (83.3)	14 (82.4)

N=161 Control Group N=175	X^2 (df), P value	2.25 (1), 0.134		0.000 (1), 1		0.007 (1), 0.932	
Maternal domestic environmental exposure							
Exposure to chemicals pre-gestation **Study Group N=161 Control Group N=175**	Yes	37 (30.6)	40 (26.1)	2 (20.0)	1 (20.0)	12 (40.0)	7 (41.2)
	No	84 (69.4)	113 (73.9)	8 (80.0)	4 (80.0)	18 (60.0)	10 (58.8)
	X^2 (df), P value	0.66 (1), 0.417		0.000 (1), 1		0.006 (1), 0.937	
Exposure to chemicals 1st trimester **Study Group N=161 Control Group N=175**	Yes	37 (30.6)	41 (26.8)	2 (20.0)	1 (20.0)	10 (33.3)	7 (41.2)
	No	84 (69.4)	112 (73.2)	8 (80.0)	4 (80.0)	20 (66.7)	10 (58.8)
	X^2 (df), P value	0.47 (1), 0.491		0.000 (1), 1		0.29 (1), 0.591	
Exposure to solvent pre-gestation **Study Group N=161 Control Group N=177**	Yes	13 (10.7)	19 (12.3)	2 (20.0)	1 (20.0)	6 (20.0)	3 (17.6)
	No	108 (89.3)	136 (87.7)	8 (80.0)	4 (80.0)	24 (80.0)	14 (82.4)
	X^2 (df), P value	0.15 (1), 0.697		0.000 (1), 1		0.04 (1), 0.844	
Exposure to solvent 1st trimester **Study Group N=161 Control Group N=177**	Yes	10 (8.3)	18 (11.6)	2 (20.0)	0 (0.0)	4 (13.3)	2 (11.8)
	No	111 (91.7)	137 (88.4)	8 (80.0)	5 (100.0)	26 (86.7)	15 (88.2)
	X^2 (df), P value	0.84 (1) 0.361		1.15 (1), 0.283		0.02 (1), 0.877	
Exposure to pesticides pre-gestation **Study Group N=161 Control Group N=177**	Yes	21 (17.4)	28 (18.1)	1 (10.0)	0 (0.0)	2 (6.7)	5 (29.4)
	No	100 (82.6)	127 (81.9)	9 (90.0)	5 (100.0)	28 (93.3)	12 (70.6)
	X^2 (df), P value	0.02 (1), 0.878		0.54 (1), 0.464		4.43 (1), 0.035	
Exposure to pesticides 1st trimester **Study Group N=161 Control Group N=177**	Yes	24 (19.8)	25 (16.1)	1 (10.0)	0 (0.0)	3 (10.0)	5 (29.4)
	No	97 (80.2)	130(83.9)	9 (90.0)	5 (100.0)	27 (90.0)	12 (70.6)
	X^2 (df), P value	0.64 (1), 0.424		0.54 (1), 0.464		2.9 (1), 0.089	
Exposure to incense pre-gestation **Study Group N=161 Control Group**	Yes	47 (38.8)	73 (47.1)	2 (20.0)	3 (60.0)	9 (30.0)	9 (52.9)
	No	74 (61.2)	82 (52.9)	8 (80.0)	2 (40.0)	21 (70.0)	8 (47.1)
	X^2 (df), P value	1.88 (1), 0.17		2.4 (1), 0.121		2.4 (1), 0.12	

N=177							
Exposure to incense in the 1st trimester **Study Group N=161 Control Group N=177**	Yes	46 (38.0)	75 (84.4)	2 (20.0)	3 (60.0)	10 (33.3)	8 (47.1)
	No	75 (62.0)	80 (51.6)	8 (80.0)	2 (40.0)	20 (66.7)	9 (52.9)
	X^2 (df), P value	2.97 (1), 0.085		2.4 (1), 0.121		0.87 (1), 0.352	
Type of maternal drinking water	X^2 (df), P value	15.6 (3), 0.001**		2.24 (1),0.134		1.68 (3), 0.642	
Type of maternal drinking water **Study Group N=158 Control Group N=175**	Tap	31 (26.1)	33 (21.4)	4 (40.0)	0 (0.0)	5 (16.7)	2 (11.8)
	Bottled	80 (67.2)	101 (65.6)	6 (60.0)	4 (100.0)	20 (66.7)	14 (82.4)
	P value	0.558		0.256		0.537	
	Well	6 (5.0)	1 (0.6)	0 (0.0)	0 (0.0)	4 (13.3)	1 (5.9)
	P value	0.094		a		0.099	
	Zamzam	2 (1.7)	19 (12.3)	0 (0.0)	0 (0.0)	1 (3.3)	0 (0.0)
	P value OR (95% CI)	0.005** 8.92 (1.92-41.52)		a		0.863	
Consanguinity **Study Group N=156 Control Group N=167**	Yes	69 (59.5)	81 (55.1)	8 (72.7)	1 (20.0)	11 (37.9)	10 (66.7)
	No	47 (40.5)	66 (44.9)	3 (27.3)	4 (80.0)	18 (62.1)	5 (33.3)
	X^2 (df), P value	0.51 (1), 0.476		3.88 (1), 0.07		3.27 (1), 0.07	
Family history **Study Group N=159 Control Group N=173**	Yes	49 (40.8)	43 (28.5)	4 (40.0)	0 (0.0)	14 (48.3)	4 (23.5)
	No	71 (59.2)	108 (71.5)	6 (60.0)	5 (100.0)	15 (51,7)	13 (76.5)
	X^2 (df), P value OR (95% CI)	5.74 (2), 0.033** 1.73 (1.04-2.89)		2.73 (1), 0.099		3.36 (2), 0.097	

*The Chi-square statistic is significant at the 0.05 level

** Homozygous common allele genotype

[a] Not possible to analyse because the groups contain zero values

If one of the cells contains zero value, the OR and 95% CI are not calculated

Appendix B

B1: Prevalence of orofacial clefts in Saudi Arabia and neighboring countries: A systematic review

SABBAGH, H. J., MOSSEY, P. A. & INNES, N. P., 2012. Prevalence of orofacial clefts in Saudi Arabia and neighboring countries: A systematic review. *Saudi Dent J,* 24**,** 3-10.

B2: Passive smoking in the etiology of non-syndromic orofacial clefts: a systematic review and meta-analysis

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Heba J Sabbagh BDS, Msc

Has no financial disclosure

Lecturer in Pediatric Dentistry Department, Faculty of Dentistry, King Abdulaziz University, Jeddah, Saudi Arabia

PhD candidate in Dundee University, Scotland, UK

hjsabbagh@gmail.com

Mona Hassan Ahmed Hassan, MBChB, MPH, PhD

Professor of Biostatistics, Department of Dental Public Health, Faculty of Dentistry Kind Abdulaziz University, Jeddah , Saudi Arabia

Department of Biostatistics, High Institute of Public Health, Alexandria University. Alexandria, Egypt.

monaha59@hotmail.com

Nicola P T Innes BDS, BMSc, BSc. PhD

Clinical Senior Lecturer in Paediatric Dentistry

University of Dundee, Dundee, Scotland, UK

n.p.innes@dundee.ac.uk

Heba M El Khodary, B.D.S., MSc, PhD.

Assistant Professor, Pediatric Dentistry Department, Faculty of Dentistry, King Abdulaziz University, Jeddah, Saudi Arabia and Lecturer of Pedodontics, Faculty of Dental Medicine (Girls' Branch), Al Azhar University, Cairo, Egypt.]

hebamelkhodary@gmail.com

Julian Little MA, PhD

JL holds a tier 1 Canada Research Chair in Human Genome Epidemiology

Department of Epidemiology and Community Medicine, University of Ottawa, Ottawa, Ontario, Canada

jlittle@uottawa.ca

Peter A. Mossey BDS, PhD

Has no financial disclosure

Professor of Craniofacial Development Associate Dean of Research

University of Dundee, Dundee, Scotland, UK

p.a.mossey@dundee.ac.uk

Abstract:

Background

Studies have found a consistent positive association between maternal smoking and non-syndromic orofacial clefts (NSOFC). However, no comprehensive assessment of the association between NSOFC and passive smoking has been undertaken. This systematic review and meta-analysis explores the relationship between maternal passive smoking and NSOFC, and compares the associations between passive and active smoking.

Methods and findings:

Search strategy, inclusion /exclusion criteria, and data extraction from studies reporting maternal passive smoking and NSOFC was implemented without language restrictions. Risks of bias in the identified studies were assessed and this information was used in sensitivity analyses to explain heterogeneity. Meta-analysis and meta-regression of the extracted data were performed. Egger's test was used to test for small study effects.

Fourteen eligible articles were identified. Maternal passive smoking exposure was associated with a twofold increase in risk of NSOFC (odds ratio: 2.11, 95% confidence interval: 1.54-2.89); this was apparent for both cleft lip with and without palate (OR: 2.05, 95% CI: 1.27-3.3) and cleft palate (OR: 2.11, 95% CI: 1.23-3.62). There was substantial heterogeneity between studies. In the studies that provided data enabling crude and adjusted odd ratios to be compared, adjustment for potential confounders attenuated the magnitude of association to about a 1.5-fold increase in risk.

Conclusion:

Overall, maternal passive smoking exposure results in a 1.5 fold increase in risk of NSOFC, similar to the magnitude of risk reported for active smoking, but there is marked heterogeneity between studies. This heterogeneity is not explained by differences in the distribution of cleft types, adjustment for covariates, broad geographic region, or study bias/quality. This thorough meta-analysis provides further evidence to minimize exposure to environmental tobacco smoke in policy making fora and in health promotion initiatives.

Introduction

Today's best evidence suggests that non-syndromic orofacial clefts (NSOFC) are multifactorial in origin involving both genetic and environmental risk factors [(1)]. Better understanding of the etiology of environmental factors can provide the basis for prevention through avoidance of exposure to risk factors.

Previous studies have been consistent in finding a positive association between active maternal smoking and NSOFC.[(2)] A meta-analysis has suggested a modest positive association between active smoking and NSOFC; for cleft lip with or without cleft palate (CL/P) the relative risk was 1.34 (95 % CI: 1.25 to 1.44) and for cleft palate (CP) relative risk was 1.22 (95% CI: 1.10 to 1.35).[(3)] Avoidance of smoking to reduce this risk is a common public health message.[(4)] However, the risk of maternal smoking exposure may be underestimated, as non-smoking pregnant women might still be exposed to passive smoking (environmental tobacco exposure) at home or work [(5)] and this is not usually taken into account, especially in developing countries. The 2014 Surgeon General's Report highlights a wide range of acute and chronic adverse health effects in infants and increased risk of adverse health outcomes resulting from second hand smoking.[(6)] The Report also noted that tobacco control measures are not sufficient to end the tobacco epidemic. Furthermore, although the association between passive smoking and congenital anomalies has been studied, the relationship has not been found to be consistent,[(7)] and no comprehensive assessment of the association between NSOFC and maternal passive smoking exposure has been undertaken. This systematic review and meta-analysis (1) in non-smoking mothers, assesses the relationship between maternal passive smoking and having an infant with NSOFC; and (2) for all mothers in the included studies, compares the associations between maternal passive smoking and maternal active smoking. We propose that

through confirmation of this relationship we will inform public health messages and support planning of community awareness programs around the adverse consequences of passive smoking during pregnancy, which is likely to be especially relevant to developing countries.

Material and methods

Search strategy and data extraction

We prepared a research protocol to investigate the relationship between orofacial clefts and passive smoking, defined as maternal exposure to environmental tobacco smoke in any location at any time during the pregnancy. The search strategy comprised key words listed separately and in combination; ((cleft lip) OR (cleft palate) OR (orofacial cleft)) AND ((passive smoking) OR (tobacco smoke pollution) OR (environmental tobacco smoke pollution) OR (smoking)). These key words were run in three search engines (PubMed, Scopus, Scholar Google) from 1980 to 2013. For Google Scholar, the search yielded 1140 articles, too numerous for review. Therefore, a modification was carried out by pooling titles from key word combinations of; ((cleft lip) OR (cleft palate) OR (orofacial cleft)) AND ((passive smoking) OR (tobacco smoke pollution) OR (environmental tobacco smoke pollution)). This revised search strategy gave a total of 1,006 articles across the three search engines: PubMed (215), Scopus (366), Google Scholar (425), (see Figure 1). The searches were run in March 2013 and did not exclude any languages. Full details of the search strategy are available in Figure S1.

The titles of all articles were reviewed by two authors independently (HJ and HK). Data presented more than once were excluded. Abstracts of articles selected on the basis of their titles were then reviewed. Articles were excluded where it was obvious from the title or abstract that the paper did not discuss the relationship of maternal passive smoking with NSOFC. The references of remaining articles were reviewed, then full texts were screened according to the following inclusion criteria;

- studies reporting passive smoking and its relationship to NSOFC ; and
- case-control, cohort or cross-sectional studies where there was a control or comparison group.

The exclusion criteria were;

- studies that discussed smoking as a whole but did not provide information on the specific association between passive smoking and NSOFC;
- studies that reported associations with genes reported to modulate the effect of smoking or gene-environmental joint effects related to NSOFC but which did not report the marginal effect of passive smoking; and
- studies that included syndromic clefts in which data on non-syndromic clefts could not be extracted.

Data extracted from these studies included;

- passive smoking definition;
- study design and setting;
- sample size, description and base population;

- prevalence and intensity of maternal passive smoking prior to pregnancy and in the first trimester for cases and controls;
- the frequency of maternal passive smoking for cleft lip with or without cleft palate (CL/P) and cleft palate (CP). Reported odds ratios (OR) and confidence intervals (CI) without frequencies were also considered; and
- prevalence and intensity of active maternal smoking.

Data were extracted, using a data extraction form (Figure S2), independently by two authors (HJ and HK). Any disagreement was resolved by discussion with a third author (MH). When possible, authors of included studies were contacted for further information on the topic. We received a response from three authors.[8-10]

Assessing risk of bias

The quality of included articles was assessed independently by two of the authors using the Newcastle-Ottawa Scale (NOS).[11] The scale measures three items; selection of cases and controls including their definition and representativeness; comparability of cases and controls in design and analysis; and exposure ascertainment. The scale has a minimum score of 0 and a maximum score of 9. Studies scoring 6 or more (correspond to 67% of the maximum score) were regarded as having a low risk of bias ("good" quality) [12]; 3–5 a modest risk of bias ("fair" quality); and studies < 3 were considered to be at substantial risk of bias ("poor" quality) Spearman's rank correlation coefficient was used to measure the degree of agreement between the authors' judgments. Disagreements were resolved through discussion. No exclusion based on risk of bias was performed. Studies were further

classified into those at substantial to modest risk of bias versus those at low risk for sensitivity analysis. Details of study quality are presented in Figure S3.

Consideration of possible small study effects

We used funnel plots to visually assess the possibility of small study effects for all studies together and also for those assessing the relationship between passive smoking and NSOFC phenotype (CL/P and CP).[13] In addition, Egger's test was used to test for small study effects.

Statistical analysis

Meta-analysis was performed using the free software Review Manager (Cochrane Collaboration).[14] The Mantel-Haenszel method was used for combining studies to calculate summary ORs and 95% CIs for passive smoking versus no smoking. To decide whether the results of the separate studies could be combined meaningfully, a statistical test of homogeneity was carried out. Based on the chi-square test, an inconsistency coefficient was computed (I^2 statistic) where a value more than 50% indicated moderate, and greater than 75% indicated high, heterogeneity [15]. Odds ratios were pooled with a fixed effect model for homogeneous studies and a random effects model for heterogeneous studies. Odds ratios with their 95% confidence limits for the individual studies and summary estimate of effect were graphically displayed in a forest plot.

For comparing the result of crude OR with the reported adjusted OR, meta-analysis was carried out using OR and standard error (SE) values that were estimated from the 95% CI.

Meta-DiSc version 1.4 (http://www.hrc.es/investigacion/metadisc_en.htm) was used to perform meta-regression for assessing the possible effect of study quality and type of cleft on the relationship between passive smoking and NSOFC. Inverse variance weights and restricted maximum likelihood estimation were used.

Sensitivity analysis

To assess stability of the results, subgroup analyses were carried out based on (a) type of NSOFC (CL/P and CP), (b) study risk of bias (NOS score >6 vs. ≤6), (c) reported adjusted OR compared to crude OR, (d) periods of measured maternal passive smoking exposure (1st trimester including and not including the pregestation period, or the pregestation period alone), (e) sequential exclusion of studies with ORs for the association between NSOFC and passive smoking greater than 3, and (f) broad geographic region in which the study was carried out (China, US and Europe, Other). These areas of subgrouping were considered likely sources of heterogeneity.

Results

The searches yielded 1,006 potentially eligible titles. After removing duplicate articles and reviewing the abstracts, the full text of 70 articles were obtained and compared to the inclusion criteria. Fifty-five articles were excluded (Figure 1) because they; did not include control group (two articles), discussed congenital anomalies in general

(two articles) or did not study passive smoking (51 articles). This resulted in 15 eligible articles (Table 1 and Figure 2). [8-10,16-27] These were all retrospective case-control studies using self-report questionnaires for non-smoking mothers.

Shaw *et al* (1996)[10] reported positive associations between specific types of NSOFC and passive smoking, defined as when a non-smoking mother frequented, worked or lived in a place where others smoked within six feet of her (for CL/P OR: 2, 95% CI: 1.2 to 3.4; for CP OR:1.6, 95 % CI: 0.7 to 3.4). However, as the study did not report the number of exposed cases and controls, it was excluded from meta-analysis, resulting in the meta-analysis being based on 14 studies. Wang *et al* (2009)[26] did not exclude smoking mothers from their analysis of maternal passive smoking. However, we included the study in our meta-analysis as this sample comprised very few mothers who reported smoking actively during pregnancy (2% in cases and 1.4% in control).

The definition of maternal passive smoking was similar in all 14 studies included in the meta-analysis. However, Li *et al* (2010)[23] defined passive smoking as the exposure of non-smoking mothers to at least one cigarette /week from a smoker in any place. Lie *et al.* (2008)[24] and Li *et al*. (2011)[22] defined it as non-smoking mother frequenting, working or living in a place where others smoked nearby, with Lie *et al.* (2008)[24] specifying a distance within two meters for at least two hours per day.

All studies measured maternal exposure to smoke during the three months of the first trimester apart from two studies which included only the first two months of the 1st trimester [22, 25]. However, seven studies compared maternal exposure to smoke for the 1st trimester combined with the pregestation period: one month pregestation;[10, 23, 26, 27] three month pregestation;[18, 20] or one year pregestation.[9, 21] (Table 1).

Honein *et al* (2007)[18] and Mirilas et al (2011)[9] measured the relationship between NSOFC and maternal passive smoking exposure in the pregestation period alone (see Table 1). Honein et al (2007)[18] have furthered analyzed the relationship with NSOFC type and found a significant relationship for CP (OR: 2.3, 95% CI: 1 to 5.3) but not with CL/P (OR: 1.3, 95% CI: 0.5 to 3.1).

The intensity of smoking was reported by two studies but with different measurement methods[23, 27]. Li et al (2010)[23] measured the number of maternal smoking exposure in hour per week, while Zhang et al (2011)[27] measured the number of hours of maternal exposure any time during one month pregestation and through the 1st trimester.

Meta-Analyses

Figure 2 shows the forest plot for the relationship between maternal passive smoking and having an infant with NSOFC in the fourteen studies with complete information on the frequency of maternal exposure to passive smoking.

There was a significant relationship between passive maternal smoking and NSOFC. The risk of having an infant with NSOFC was doubled following maternal exposure to environmental tobacco (OR: 2.11, 95% CI: 1.54 to 2.89). When studies with ORs greater than 3 were excluded, the relationship continued to be significant; after excluding the study of Mirilas et al (2011)[9] the OR was 2.04 (95% CI: 1.49 to 2.8); after excluding that of Jia et al (2011)[19] in addition to that of Mirilas et al (2011)[9] the OR was 1.8 (95% CI: 1.4 to 2.32); after excluding the study of Zhang et al (2011)[27] in addition to these two studies, the OR was 1.66 (95% CI: 1.34 to 2.06); and after excluding all studies with an OR greater than 3, the OR was 1.55 (95% CI: 1.28 to 1.87).

Figure 3 shows the forest plot for the relationship between maternal smoking and having an infant with NSOFC in studies that reported passive smoking and active smoking. Maternal active smoking and passive smoking was reported by eleven studies and found to be significantly related to NSOFC; OR: 2.07, 95% CI: 1.42 to 3.01 for passive smoking; and OR: 1.5, 95% CI: 1.17 to 1.93 for active smoking. Although the OR=1.5 for active smoking was less than the OR=2.07 for maternal passive smoking, the difference was not statistically significant (P= 0.17).

Figure 4 included studies that reported adjusted OR for the association between NSOFC and passive smoking. Both meta-analysis; for the crude OR and the reported adjusted OR found a significant relationship between NSOFC and passive smoking (OR: 1.79, 95% CI: 1.34 to 2.4 and OR: 1.54, 95% CI: 1.11 to 2.12) respectively. In addition, there were no significant differences between the two meta-analyses (P= 0.49). The factors for which adjustment was made in each study are listed in Table 1 and differed between studies.

Figure 5 shows forest plot for meta-analysis of the association between maternal passive smoking exposure period and NSOFC. The period of maternal exposure was divided into two groups; studies reporting maternal passive smoking exposure during the 1st trimester including or not including the pregestation period and studies reporting maternal passive smoking exposure prior to pregnancy alone. NSOFC was significantly associated with maternal exposure to passive smoking during the first trimester period including or not including pregestation period (OR: 2.03, 95% CI: 1.49 to 2.76). However, no significant relationship was found for maternal exposure prior to pregnancy alone (OR 1.62, 95% CI: 0.93 to 2.82). The difference between the two periods was not statistically significant (P= 0.49).

Figure 6 shows the relationship between passive smoking and the different types of NSOFC; CL/P and CP. The risk of having an infant with either CL/P or CP associated with passive smoking was approximately doubled (for CL/P OR: 2.05, 95% CI: 1.27 to 3.3; for CP OR: 2.11, 95% CI: 1.23 to 3.62).

Papers assessing the relationship between maternal smoking and having an infant with NSOFC were also sub-grouped according to region (China, United State and Europe, and other countries) (Figure 7). There was a significant difference between the three regions (P= 0.01) with a higher OR (OR: 3.08, 95% CI: 1.96 to 4.87) for China than the other two regions. However, there remained high heterogeneity between studies within China, and within the US and European group.

The magnitude of association between maternal passive smoking and NSOFC was significantly higher (P= 0.0003) in the studies assessed as of "fair" or "poor" quality than in the other studies. However, differences in study quality and risk of bias do not account for the substantial heterogeneity of effect between studies (Figure 8). Each meta- analyses showed significant heterogeneity with I^2 more than 75% for the majority of subgrouping.

Assessing risk of bias

Figure 8 shows the included studies distributed according to NOS risk of bias scores. Out of the 14 studies, 11 had a low risk of bias score[8, 9), (16-18), (20, 21), (23-26)] (OR: 1.57, 95% CI: 1.26 to 1.96); while three of the studies were rated being at moderate to high risk [19, 22, 27)] (OR: 4.92, 95% CI: 2.74 to 8.81). The main reason for the lower NOS in some studies was lack of comparability and matching. There was a significant

difference in the maternal passive smoking risk OR between these two groupings (P= 0.0003).

Sensitivity analysis

The sensitivity analysis demonstrated stability and reliability of the meta-analyses results through consistency of meta-analyses results, between different study subgroupings; the significant relationships between maternal passive smoking and NSOFC persisted in all of the situations evaluated (Figures 3, 4, 5, 6 and 7).

Meta regression

Table 2 shows that the association between passive smoking and NSOFC varies by study quality, region but not cleft type. The magnitude of the association is lower in studies appraised as of good quality than in other studies and higher for Asian studies.

Evaluation of small study effects

Figure 9 shows the funnel plots for all studies together assessing the relationship between NSOFC and passive smoking. Figure 9 shows studies assessing the relationship between passive smoking and NSOFC phenotype (CL/P and CP). Though the graph did not have the shape of a funnel it is almost symmetrical around the central line, indicating absence of small study effect. No statistically significant small study effect was detected by Egger's test (to further assess small study effect), either for all studies together and for studies of specific cleft types (P>0.05).

Discussion

This systematic review and meta-analysis found around a twofold increase in the risk of NSOFC associated with environmental tobacco exposure. Not all studies adjusted for potential confounding factors, and in those that did, the covariates differed between studies. In the studies that presented both crude and adjusted estimates of effect, there was a modest attenuation of the magnitude of association (OR reduced from (1.79 to 1.54) and it was noteworthy that the magnitude of association in studies appraised as having a low risk of bias was about 1.5. Overall, the magnitude of association was similar between CL/P and CP, but there was substantial heterogeneity between studies.

Prevention and protecting against having an infant with NSOFC could be possible through understanding the associated risk factors. In 2014, the Surgeon General considered the evidence in the literatures to be sufficient to infer a causal relationship between maternal smoking in early pregnancy and orofacial clefts [(6)].

However, the effects of passive smoking have not been fully evaluated and a single study would provide insufficient evidence [(28)]. In addition, passive smoke could have a more potent adverse effect on infants in a domestic environment as pregnant women and nursing mothers might be unaware of its existence or its importance as a risk factor. In some countries, smoking has been prohibited in enclosed public places to protect non-smoking individuals from passive smoking. However, many other countries, both developing and some developed countries, have not introduced smoking restriction legislation [(29)]; and mothers may be exposed to passive smoke in the domestic environment due to heating and cooking as well as tobacco smoke.

Environmental smoking exposure is difficult to measure. The main methods of assessment are self-report and biochemical assays of nicotine or cotinine [30]. All studies included in this meta-analysis used self-reported questionnaires. Kvalvik *et al* (2012)[30] validated maternal self-reported tobacco use during pregnancy with plasma cotinine in the Norwegian Mother and Child Cohort Study. They concluded that self-reported tobacco use was a valid marker for tobacco smoke exposure including passive smoking. In addition, Salmasi *et al* (2010)[31] compared the results of the association between perinatal outcomes and maternal self-reported exposure to passive smoking and biochemical analysis (2–10 ng/ml of cotinine) and found similar findings with both methods of exposure assessment.

Eleven out of the 14 studies included in the meta-analysis presented data on maternal active smoking as well as on the effects of passive smoking in non-smoking mothers. In aggregate, passive smoking increased the risk of NSOFC (OR: 2.07, 95% CI: 1.42 to 3.01) more than active smoking (OR: 1.5, 95% CI: 1.17 to 1.93), although this difference was not statistically significant (P= 0.17) and there was considerable heterogeneity between studies. Given that active smokers are exposed both to direct inhalation and to sidestream smoke, a stronger association for passive smoking than for active smoking is unexpected. A possible explanation for this finding could be under-reporting of active smoking as the studies rely on self-report and active smoking mothers may be compelled to under-report their smoking because of the associated stigma. This sense of shame would not influence reporting of the passive smoking reports to the same extent. Another explanation or contributory factor could be that duration/ dose response might influence the high association for passive smoking compared to active smoking; mothers may be exposed to passive smoke under occupational circumstances which would lead to a longer duration of exposure.

However, there was insufficient detail in the reports to be able to extract this level of data.

Such a difference might be due to active smoking mothers tending to stop smoking when they are pregnant but being unaware that environmental tobacco exposure is harmful to the health of the developing infant. Therefore, public health awareness and further studies on passive smoking are important.

Our search focused on manuscripts of passive smoking studies. Whilst active smoking results are reported in some of these passive smoking studies' papers, our results do not represent a complete picture of the active smoking literature. However, active smoking has only been used for the purpose of comparison in assessing the passive smoking effect In order to probe the association between passive smoking and NSOFC further, in the eleven studies that reported adjusted OR, we compared the adjusted OR and crude OR. Although the differences between the estimates were not formally significant (P= 0.49), there was some attenuation of the OR by adjustment.

We found a higher risk of association between NSOFC and maternal passive smoking in the 1st trimester including or not including the pregestation period (OR: 2.03, 95% CI: 1.49 to 2.76) compared to those who were exposed to environmental tobacco only in the period prior to pregnancy (OR: 1.62, 95% CI: 0.93 to 2.82) but the difference between the two periods is not statistically significant (P= 0.49). This could indicate that if mothers were exposed to passive smoking prior to pregnancy, there is still an opportunity to protect their embryo from NSOFC through avoiding passive smoking. However, this finding needs more investigation as there were only two studies that reported maternal passive smoking exposure prior to pregnancy.

The magnitude of association (OR=4.92) in the three studies with a NOS lower than 6 was significantly higher odds ratio than for the studies with a NOS scoring 6 or more. These studies were all carried out in China, which could in part explain the significant difference between the three different regions (Figure 7), with the higher OR in studies carried out in China (OR: 3.08) compared to other regions (OR: 1.39 for the studies in Europe and US; and OR: 1.54 for the studies in Iran and Brazil). It is noteworthy that in all but one[23] of the Chinese studies, the prevalence of reported passive smoking in control mothers was substantially lower than has been reported in pregnant women [32]or women of reproductive age[33, 34] in large surveys in China.

The two categories associated with low NOS study scoring were "exposure ascertainment" and "comparability". The ascertainment of exposure was affected because information on smoking was gathered through questionnaire or interview, which is not usually considered to provide optimal information on smoking, although there is evidence that suggests that self-reported passive smoking gives information of comparable quality to cotinine assessment.[30] The comparability between cases and controls in terms of minimizing the effects of potential confounding by means of matching and/or adjustment for confounding variables was also limited in studies with low quality (Figure S3). Therefore, we strongly advise careful consideration of the comparability of source populations in recruiting cases and controls in future studies.

Further research on the intensity, duration of exposure and agreement on standardized methods for recording and reporting will aid further investigation of this environmental hazard.

Tobacco use is rapidly increasing among women of reproductive age in many countries because they are actively targeted by tobacco marketing campaigns [35, 36] and this would be likely to result in an increase in the prevalence of exposure to environmental tobacco smoke. In the U.S., a decline in the prevalence of exposure to passive smoking from the late 1980s has leveled off since about 2002 [37]. There also continues to be substantial exposure to passive smoking in Canada [38]. In England, the impact of smoke-free legislation on exposure to passive smoking was greater than the underlying long-term decline in exposure, demonstrating a positive effect of legislation [39]. An increased risk of cleft palate associated with passive smoking has been mentioned in a paper making a case for a worldwide ban on smoking in public places [40]. Therefore, we suggest that the results of the present meta-analysis provide a more solid basis to argue for interventions to minimize exposure to environmental tobacco smoke in policy making fora and in health promotion initiatives.

Conclusion

In studies that adjust for potential confounding and/or are adjudged to have low risk of bias, maternal passive smoking exposure is associated with approximately a 1.5 fold increase in the risk of having an infant with NSOFC. There is marked heterogeneity between studies, which is not explained by differences in the distribution of cleft type, adjustment for covariates, difference in regions, or study quality. This thorough meta-analysis provides further evidence to argue for interventions to minimize exposure to environmental tobacco smoke in policy making fora and in health promotion initiatives.

Acknowledgment

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- JL holds a tier 1 Canada Research Chair in Human Genome Epidemiology

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List of Figures:

Table 1.Characteristics of studies of risk of NSOFC in the offspring of non-smoking mothers exposed to passive smoking included in meta-analysis

Reference	Site and Country	Duration of data collection	Study design	Total Sample size (smoking+ non-smoking) mothers	Reported period of maternal exposure	Non-smoking mothers exposed to passive smoking/ total non-smoking mothers (%)			Reported adjusted OR (95% CI) for passive smoking with adjusted factors	Active smoking mothers/total sample size (%)	
						NSOFC	Type of NSOFC (CL/P and CP)	Controls		Cases	Control
Beaty *et al* (2001)[16]	Treatment centers, Maryland craniofacial clinics, Children's National Medical Centre , Washington DC, **US**	1992-1998	Case-control	171 cases 182 control	1st trimester	24/107 (22.4)	CL/P: 14/73 (19.2) CP: 10/34 (29.4)	18/130 (13.8)	CL/P:1.04 (0.067-1.62) CP: 1.17 (0.68-2.02) Maternal age and education	27/171 (15.8)	25/182 (13.7)
Chevrier *et al* (2008)[17]	Maxillofacial departments in; Lyon; Grenoble; Rhône-Alpes region; Paris; Clermont-Ferrand; Auvergne, **France**	1998–2001	Matched case-control (age, sex, origin, place of residence	240 cases, 236 controls	1st trimester	97/173 (56.1)	CL/P: 65/119 (54.6) CP: 32/54 (59.3)	70/167 (41.9)	1.8 (1.2-3.4) Region and Child sex	67/240 (27.9)	69/230 (30)
Honein *et al* (2007)[18]	Coordinated by the Centers for Disease Control and Prevention (CDC). Eight Centers for Birth Defects Research and Prevention contributed data: Arkansas, California, Iowa, Massachusetts, New Jersey, New York, Texas, and CDC (Atlanta,GA), **US**	___	Population based, multicenter, matched case-control (site, frequency of births per month) Random sample of live births in controls:	933 CL/P 528 CP 3390 control	Three month pregestation + 1st trimester Pregestation 1st trimester	235/1104 (21.3) 14/1104 (1.3) 13/1104 (11.4)	CL/P: 147/699 CP:88/528 (22) CL/P:7/699(1) CP:7/405(1.7) CL/P: 9/6991 (0.1) CP: 4/405 (0.99)	554/2699 (20.5) 21/2699 39/2699 (1.4)	1.1 (0.09-1.3) Child sex, folic acid exposure, maternal age, ethnicity, gravidity 1st degree relative with birth defect were excluded	352/1461 (24.1)	684/3390 (20.2)
Jia *et al* (2011)[19]	West China College of Stomatology, Sichuan University, Department of Cleft Lip and Palate Surgery, **China**	2008 and 2010.	Hospital based, Case–control	537 CL/P 176 CP 221 controls	1st trimester	402/713 (56.4)	CL/P: 302/537 (56.2) CP: 100/176	27/221 (12.2)	11.42 (6.87-19) Child sex, birth weight, maternal	18/713 (2.5)	2/221 (0.9)

							(56.8)		age, and weight, multi-vitamins, calcium and folic acid exposure		
Jianyan *et al* (2010)[20]	**China**	___	Hospital-based, matched case-control (sex age, socio-economic status)	200 CL/P 200 controls	Three month Pregestation + 1st trimester	121/200 (60.5)		87/200 (43.5)	1.72 (1.08-2.74 Maternal and paternal schooling	a	a
Leite and Koifman (2009)[21]	city of Rio de Janeiro **Brazil**	___	Hospital-based, matched case–control (sex, age, location of parents resident)	274 cases 548 controls	One year pregestation + 1st trimester	166/274 (60.6)		281/548 (52.3)	1.48 (1.09-2.01) Maternal education, age and alcohol intake	68/274 (24.8)	94/548 (17.1)
Li *et al* (2010)$_{22}$	Data from a population-based case-control study of external malformations in 4 counties (Pingding, Xiyang, Taigu, Zezhou) of Shanxi Province,[38] **China**	2003-2006	Population-based Matched case–control (county, sex, maternal ethnic, conception date)	88 cases (CL/P) 651 controls	One month pregestation + 1st trimester	59/88 (67) 1-6 times/week:31/88 (35.2) >6times: 28/88(32)		348/651 (54) 1-6 times/week 234/651(35.9) >6times: 114/651 (17.5)	CL/P: 2 (1.2-3.4) Maternal occupation, fever and flu pregestation, child sex	a	a
Li *et al* (2011)[23]	Study: College of Stomatology, West China Control: Women's and Children's Hospital, West China, **China**	Study: 2005-2008 Control:2006 - 2007		162 cases 304 control		69/162 (42.6)		54/304 (17.4)		a	a

Lie *et al* (2008)[24]	**Norway**	1996-2001	Matched case–control (time) random selected control	573 cases 763 controls	1st trimester	90/334 (26.9)	1st trimester CL/P: 58/210 (27.6) CP:32/196(16.3)	106/520 (20.4)	CLP: 1.59 (1.02-2.47) CP: 1.05 (0.55-2) Maternal education, occupation, alcohol intake, folic acid supplement, diet and multivitamins, paternal income, child date of birth	239/432 (55.3)	243/763 (31.8)
Little *et al* (2004)[8]	Scotland, Manchester, Merseyside **UK**	1997-2000	Population-based Matched case-control (sex, date of birth, region)	190 cases 248 controls	1st trimester	67/110 (60.9)	1st trimester CL/P: 40/76 (52.6) CP 27/78 (34.6)	111/189 (58.7)	1 (0.6-1.6) Child sex, season of birth maternal education, ethnicity	80/190 (42.1)	59/248 (23.8)
Mirilas *et al* (2011)[9]	Pediatric Surgery Department, **Greece**	2004 & 2009	Residency Matched case-control	35 case control 35 matched (place)	One year pregestation or 1st trimester One year pregestation + 1st trimester Pregestation:	34/35 (97.1) 16/35 (45.7) 18/35 (51.4)	CL/P: 16/35 (45.7)	25/35 (71.4) 11/35 (31.4) 14/35 (40)		15/35 (42.9) 9/53 (17)	20/35 (57.3) 7/35 (20)
Taghafi *et al* (2012)[25]	Bahrami Hospital, Tahran, **Iran**	2005-2010	Hospital base Case-control	300cases 300 controls	Three month Pregestation + 1st	113/300 (37.7)	CL/P 113/300 (37.7)	80/ 300 (26.7)	0.613 (0.43-0.87) Child sex, maternal age, education,	7/300 (2.3)	5/300 (2)

					trimester				socioeconomic state, iron exposure, vitamin use, medication, smoking X-ray exposure, consanguinity		
Wang *et al* (2009)[26]	Thirteen districts and counties, Shenyang, **China**.	2000 to 2007	population-based control matched (gender, place, date of birth) (2 control for each case)		One month Pregestation + 1st trimester	168/ 586 (28.7)		192/ 1172 (16.4)	2.05 (1.47-2.87) Maternal age and weight	12/586 (2)	16/1172 (1.4)
Zhang *et al* (2010)[27]	Centre for the Rehabilitation of Craniofacial Anomalies, Harbin Medical University, Harbin, **China**	2006-2009	Case-control Not matched	304 cases CLP140 CP77 CL86 453 controls	One month Pregestation + 1st trimester	224/323 (69.3)	CL:79/106 (74.5) CLP: 96/140 (68.6) CP 49/77(63.6)	169/454 (37.2)		14/300 (4.7)	6/545 (1.1)

a. missing information.

Table 2: Univariate meta-regression analysis relating cleft type and study quality to effect size estimates of the relation between passive smoking and NSOFC

Variable	Coefficient	Standard error	p value	Relative odds ratio (95% CI)	Tau-squared
Constant	0.579	0.636	0.379	----	
Cleft type	0.132	0.411	0.752	1.14 (0.47 to 2.77)	0.566
Constant	1.579	0.188	0.000	----	
Quality	-1.144	0.214	0.000	0.32(0.20 to 0.51)	0.067
Constant	0.221	0.067	0.007	----	
Region	0.407	0.134	0.011	1.50(1.12 to 2.02	0.090

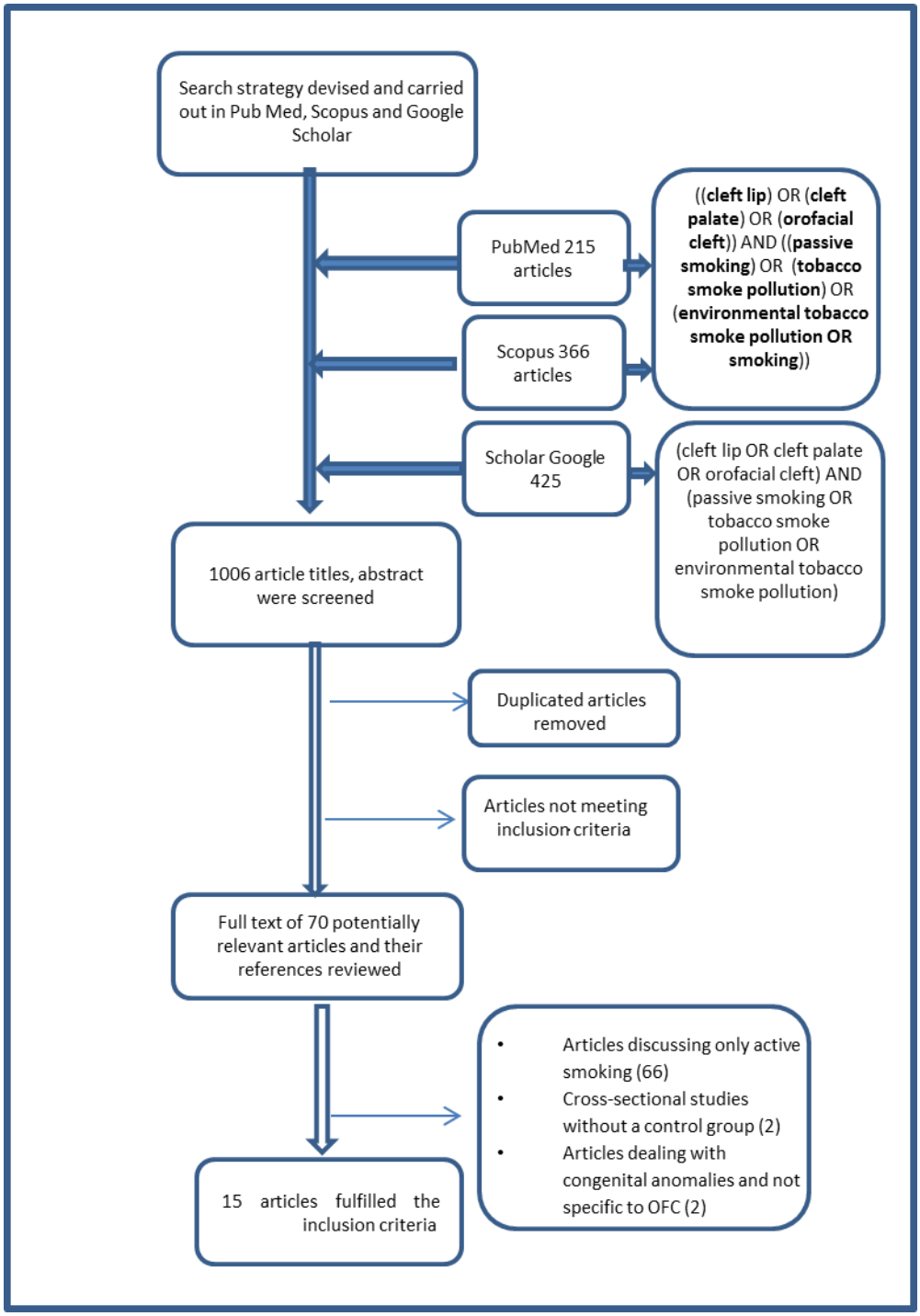


Figure 1: Flow diagram of study selection process.

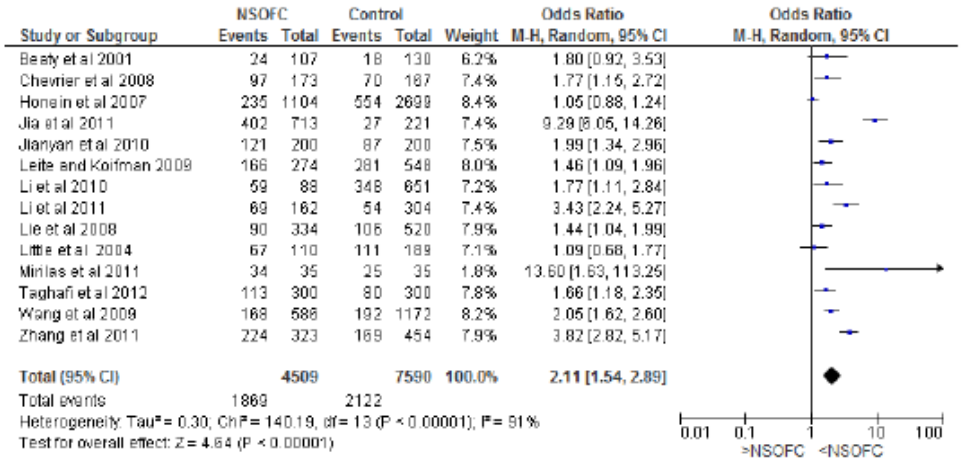
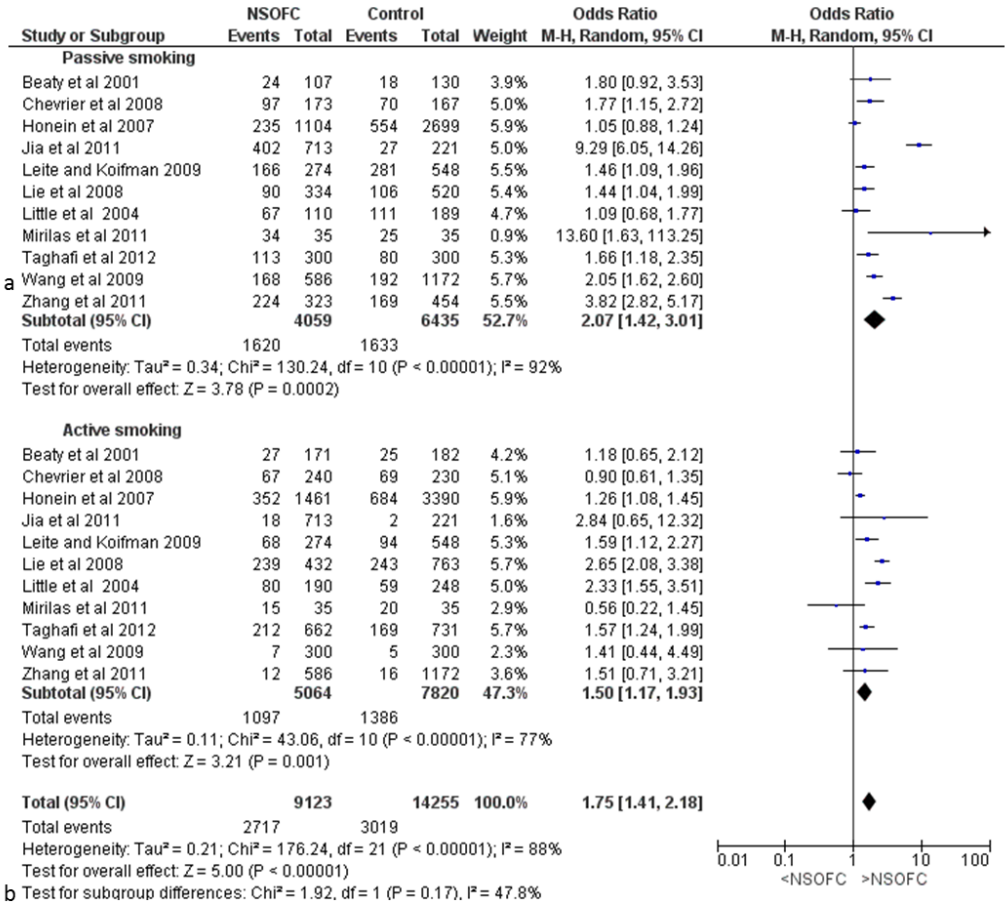


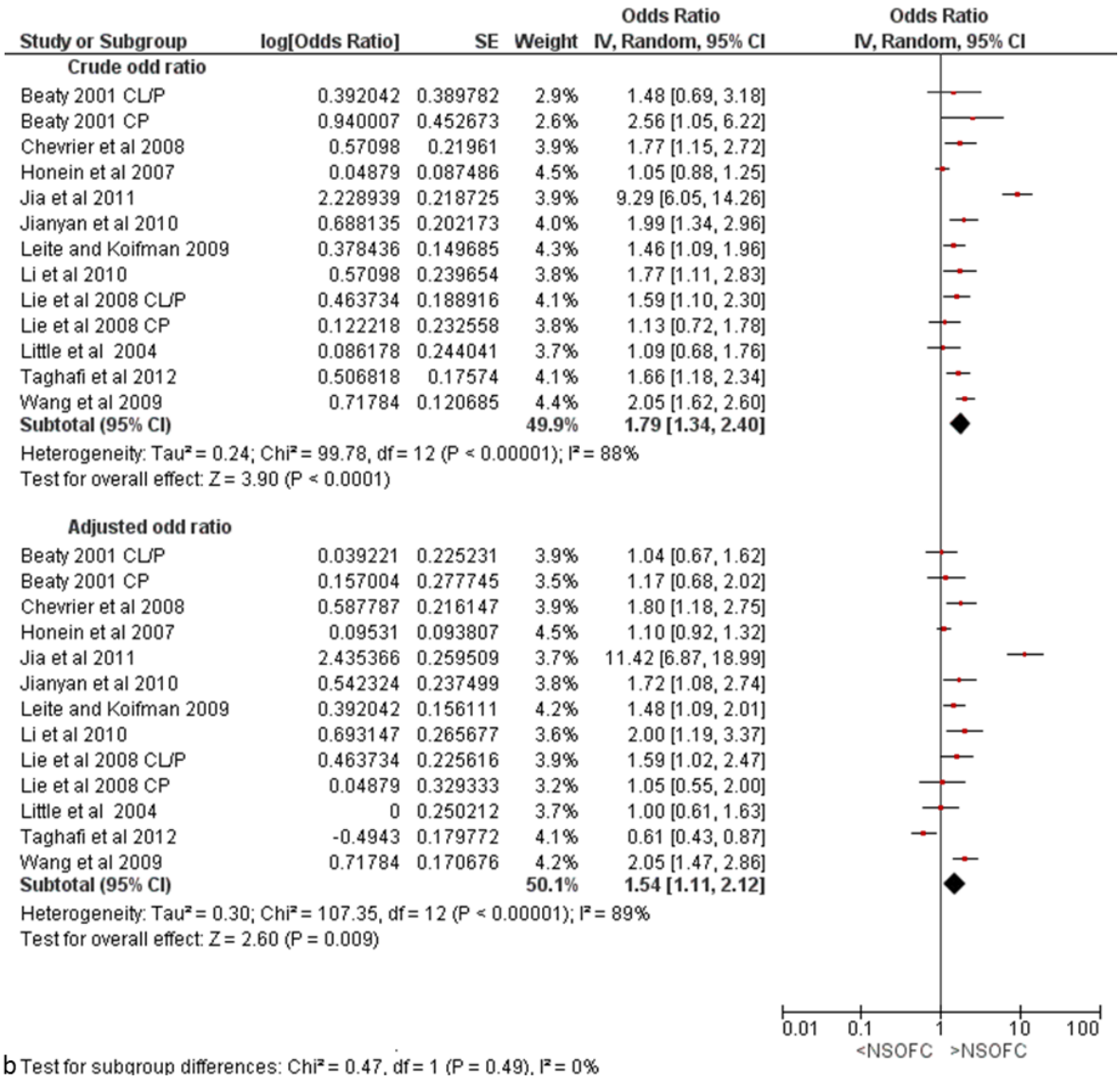
Figure 2: Forest plot for meta-analysis of the association between maternal passive smoking and the risk of having an infant with NSOFC



a. Wang did not exclude Active smoking mothers but they were less than 2%.

b. Test of subgroup differences for the differences between active and passive maternal smoking

Figure 3: Forest plot for meta-analysis of the association between maternal passive smoking and the risk of having an infant with NSOFC, comparing the different types of maternal smoking exposure (active and passive)



b . Test of subgroup differences for the differences between CL/P and CP

Figure 4: Forest plot for meta-analysis showing the crude and reported adjusted OR for the association between maternal passive smoking and NSOFC

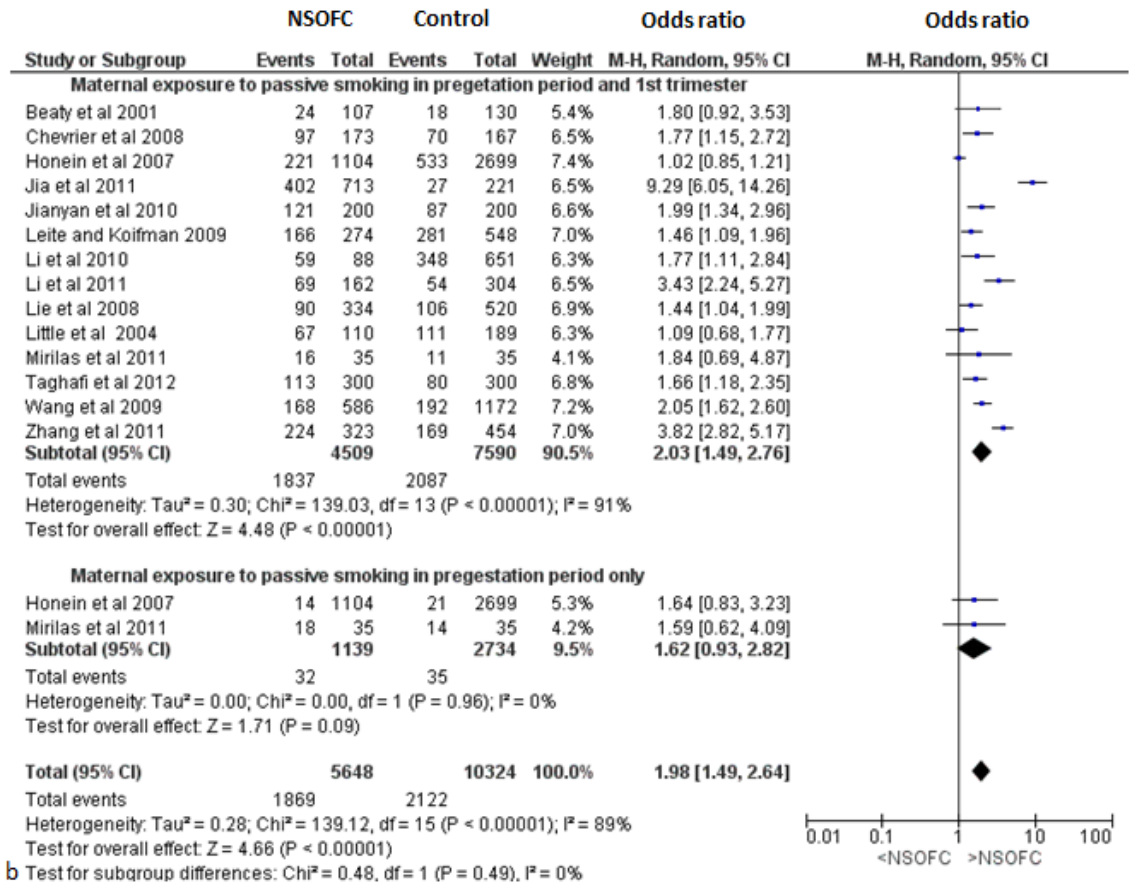
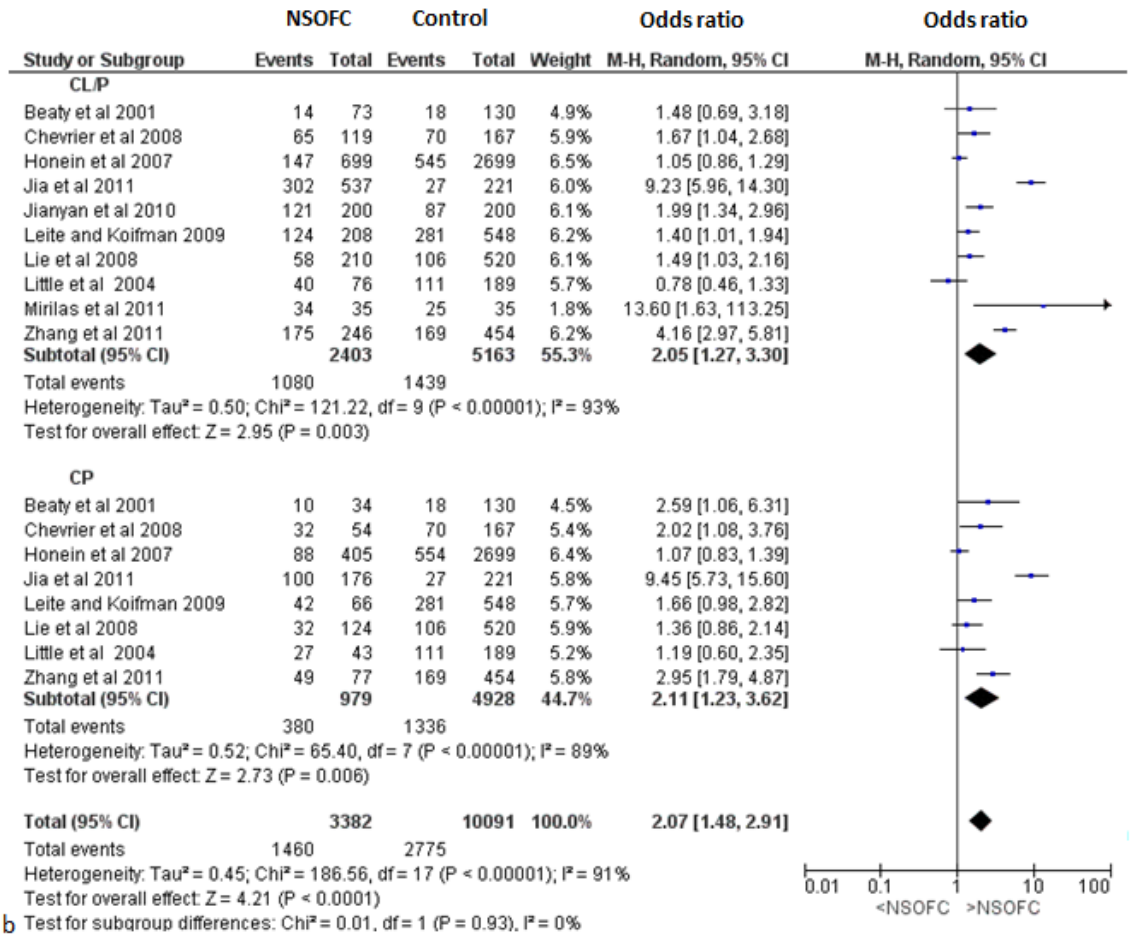
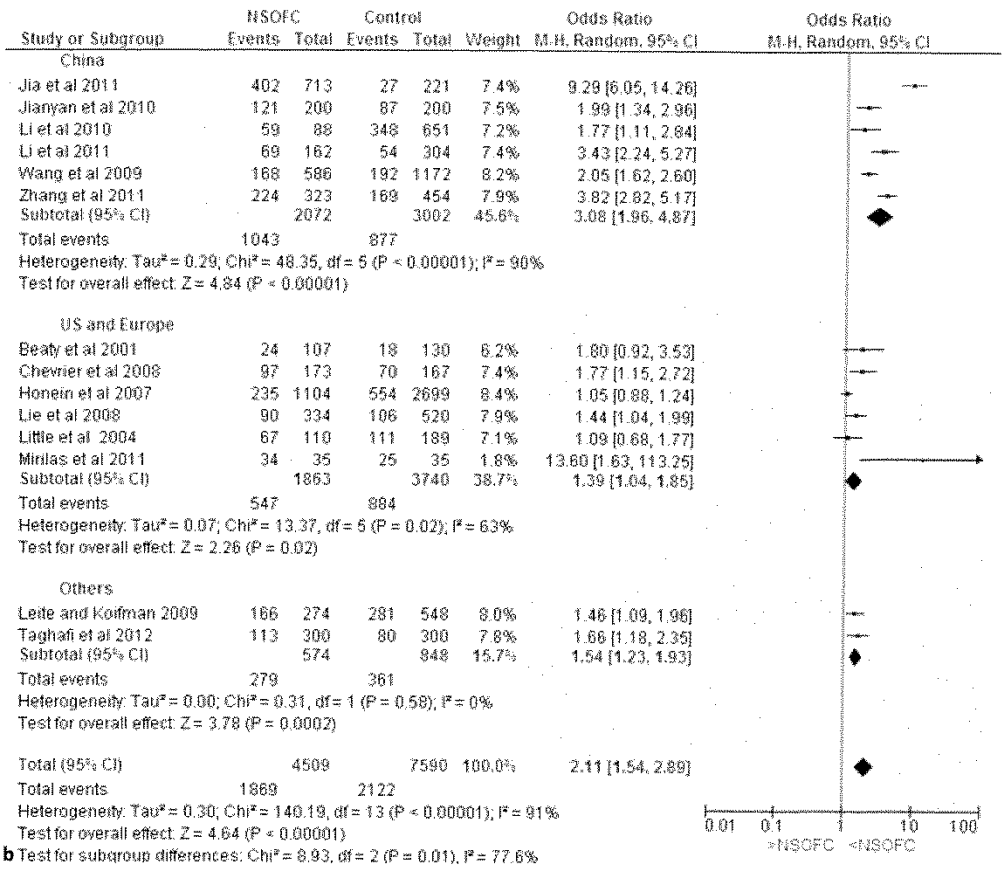


Figure 5: Forest plot for meta-analysis of the association between NSOFC and maternal passive smoking exposure in the 1st trimester including or not including the pregestation period compared to maternal exposure prior to pregnancy period alone.



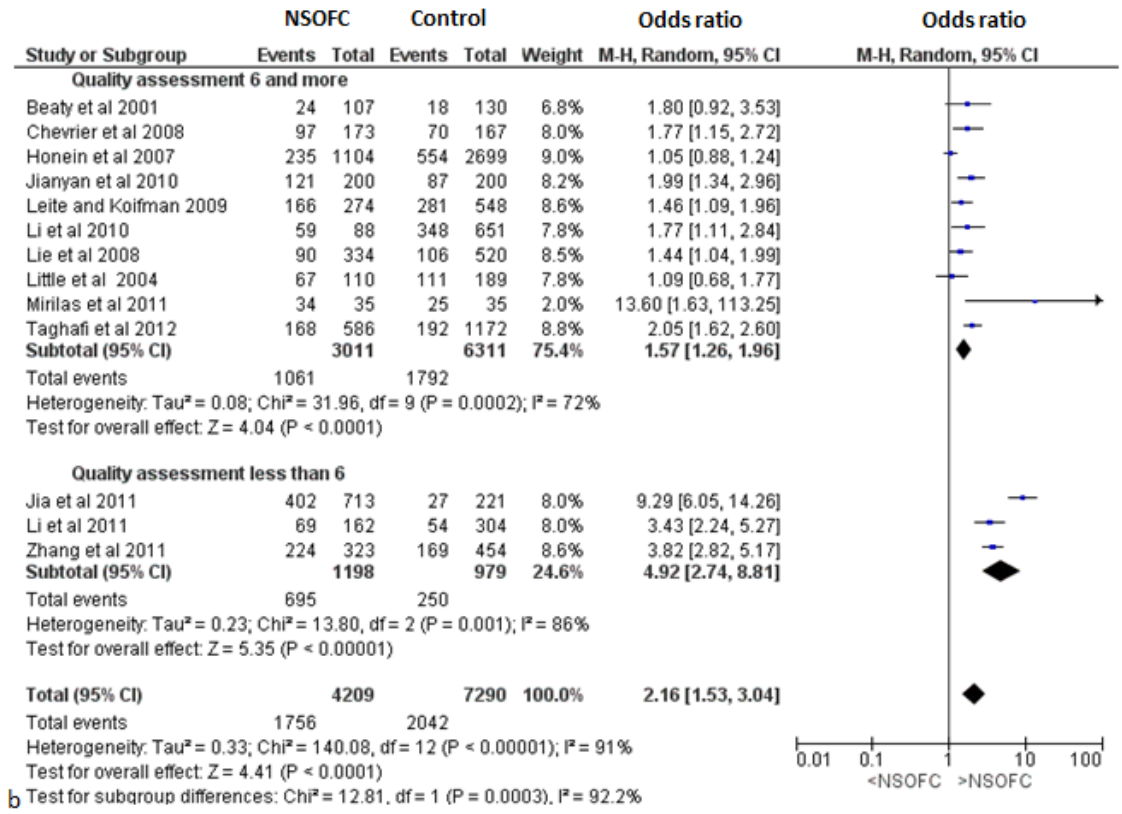
b. Test of subgroup differences for the differences between CL/P and CP

Figure 6: Forest plot for meta-analysis of the association between NSOFC phenotype (CL/P and CP) and maternal passive smoking



b . Test of subgroup differences for the differences between different regions

Figure 7: Forest plot for meta-analysis of the association between maternal passive smoking and NSOFC according to region



b. Test of subgroup differences for the differences between studies with quality score <6 and those with quality score 6 or more.

Figure 8: Forest plot for meta-analysis of the association between maternal passive smoking and NSOFC according to study quality (NOS scale)

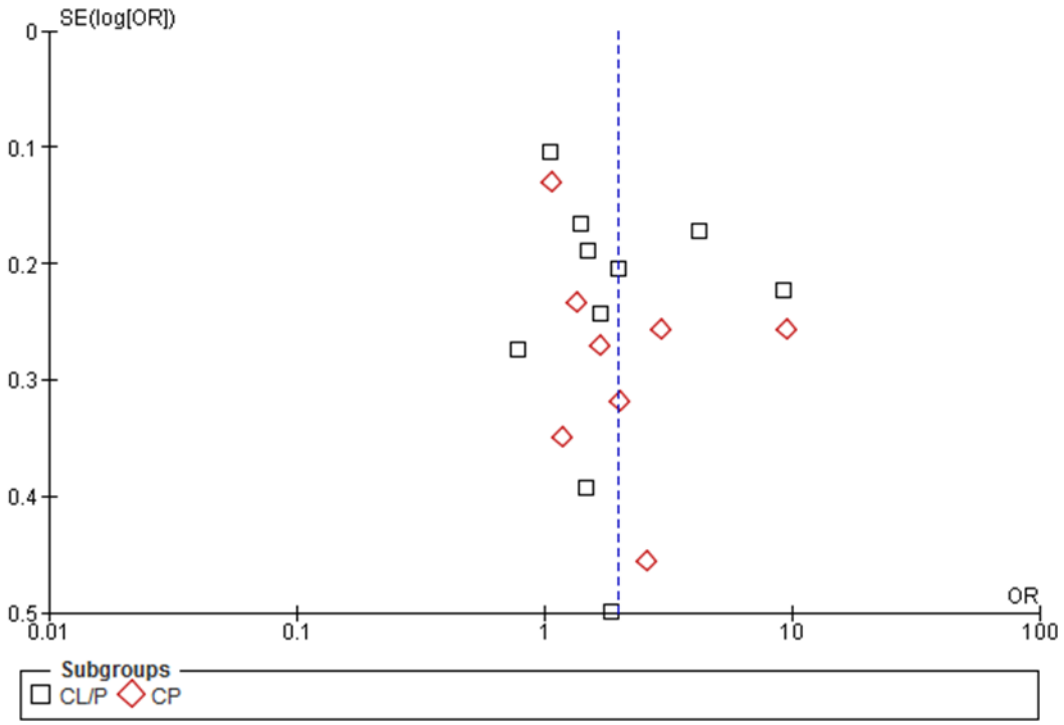


Figure 9: Funnel plot for studies showing the relationship between passive smoking and both CL/P and CP.

B3: Parental consanguinity and non-syndromic orofacial clefts in children; a systematic review and meta-analyses

SABBAGH, H. J., HASSAN, M. H., INNES, N. P., BAIK, A. A. & MOSSEY, P. A., 2014. Parental consanguinity and nonsyndromic orofacial clefts in children: a systematic review and meta-analyses. *Cleft Palate Craniofac J,* 51**,** 501-13.

B4: Prevalence and characteristics of non-syndromic orofacial clefts in Jeddah, Saudi Arabia and the influence of consanguinity

ALAMOUDI, N., SABBAGH, H. J., INNES, N., EL DERWI, D., HANNO, A., AL-AAMA, J., HABIBALLAH, A. H. & MOSSEY, P. A. 2014. Prevalence and characteristics of non-syndromic orofacial clefts and the influence of consanguinity. *Journal of Clinical Pediatric Dentistry,* 38**,** 241-246.

B5: Epidemiology of non-syndromic orofacial cleft (NSOFC) in Medina, Saudi Arabia

ABDULHAMEED, F. D., SABBAGH, H. J., HUMMAIDA, T. I. & ALAMOUDI, N. M. 2014. Epidemiology of non-syndromic orofacial cleft (NSOFC) in Medina, Saudi Arabia. *Experimental and Clinical Cardiology,* 20**,** 505-516.

B6: Prevalence of non-syndromic orofacial clefts in Saudi Arabia

Prevalence of non-syndromic orofacial clefts in Saudi Arabia and the effects of parental `consanguinity

مدى انتشار الشفة الأرنبية في المملكة العربية السعودية وتأثير زواج الأقارب عليها

HJ SABBAGH, NPT INNES, B SALLOUT, NM ALAMOUDI, M AL-HAMDAN, N AL-HAMLAN,

AI AL-KHOZAMI, F DAOOD, JY AL-AAMA, PA MOSSEY.

Running title: Nonsyndromic OFC in Saudi Arabia

From the Division of Oral Health Sciences and WHO Collaborating Centre (Sabbagh, Innes, Mossey), University of Dundee, Park Place, DD1 4HR, United Kingdom, the Preventive Dental Sciences Department (Sabbagh, Alamoudi), Pediatric Dentistry, Faculty of Dentistry, King Abdulaziz University, the Maternal-Fetal Medicine Department (Sallout), Women's Specialized Hospital, King Fahad Medical City, Riyadh, the Maxillofacial Surgery Department (Al-Hamdan), King Saud Medical City, Ministry of Health, Orthodontic Division, King Saud bin Abdulaziz University for Health Sciences, the Orthodontic Department (Al-Hamlan), and the Pediatric Department, Nursing Services (Al-Khozami), King Abdulaziz Medical City-Riyadh, National Guard Health Affairs, Pediatric Surgery (Daood), Maternity and Children's Hospital, Ministry of Health and the Genetic Medicine Department (Al-Aama), Faculty of Medicine, Princess Al-Jawhara Albrahim Centre of Excellence in Research of Hereditary Disorders, King Abdulaziz University Hospital, Saudi Arabia.

Address correspondence and reprint request to:

Heba J Sabbagh

PhD candidate, Division of Oral Health Sciences, University of Dundee, Scotland

Specialist in pediatric Dentistry, King Abdulaziz University, Saudi Arabia

Address: University of Dundee, Park Place, DD1 4HR, United Kingdom

00966 0505668481

hjsabbagh@gmail.com

Abstract

Objectives: Non-syndromic orofacial clefting (NSOFC) is the most prevalent craniofacial birth defect worldwide with geographic and ethnic variation. We describe the characteristics and prevalence of NSOFC in Riyadh and in three main cities of Saudi Arabia together, and assess the effects of parental consanguinity on NSOFC phenotypes.

Methods: All infants (114,035) born at three referral centers in Riyadh and six hospitals in Jeddah and Medina from January 1, 2010 to December 31, 2011 were screened. NSOFC cases (133) were identified and data were collected from clinical examination records and parent interviews. A NSOFC diagnosis was confirmed by reviewing medical records and contacting the infants' pediatricians. Infants (233) matched for sex and born in the same hospitals during the same period, were selected as controls to further analyze the effects of consanguinity on NSOFC.

Results: The prevalence of NSOFC was 1.07/1000 births in Riyadh, and 1.17/1000 births overall; cleft lip (CL) was 0.47/1000 births, cleft lip and palate (CLP) was 0.42/1000 births, and cleft palate (CP) was 0.28/1000 births. CP was significantly associated with consanguinity (P= 0.047, OR: 2.5, and 95% CI: 1 to 6.46), particularly for 1st cousin marriages (72.7% for NSOFC compared to 58.9% for controls).

Conclusion: The prevalence of NSOFC in Riyadh alone, and in the three main cities in Saudi Arabia, was marginally lower than the mean global prevalence (1.25/1000 births). CL was higher than CLP, in contrast to the global pattern.

Introduction

Non-syndromic orofacial clefting (NSOFC), including isolated cleft lip (CL), cleft lip and palate (CLP), and isolated cleft palate (CP), is the most common craniofacial defect worldwide with an estimated mean global prevalence of 1.25/1000 live births (1). However, the prevalence of NSOFC varies geographically and across different ethnic groups (2). Although the ethnicity of the Middle East is considered Caucasian (3, 4), geographically it is located between three continents (Asia, Africa and Europe) which makes it unique and, in reality, a mixture of three ethnicities. A small number of studies have measured the prevalence of NSOFC in Middle East countries with the reported prevalence ranging from 0.3 to 2.19/1000 births (5-9) In addition, consanguineous relationships have been suggested to increase the prevalence of congenital anomalies, particularly for recessive gene disorders (10).

Saudi Arabia, one of the largest countries in the Middle East, has a high rate of consanguineous marriage that varies between regions. Thus, the aim of this study was to (1) describe the characteristics and prevalence of NSOFC (CL, CLP, and CP) in Riyadh (the capital city in the central region of Saudi Arabia) and (2) describe the prevalence of NSOFC phenotypes and its relationship to consanguinity in Saudi Arabia.

Methods

Subjects

This study was conducted at three medical referral hospitals in Riyadh: King Fahad Medical City, King Saud Medical City, and Riyadh National Guard Hospital. Riyadh is the capital city of Saudi Arabia and constitutes 25% of the Saudi population {MINISTRY OF ECONOMY AND PLANNING, 2013 #348). Data from two previously published studies, conducted in Jeddah and Medina, were

also included(11, 12) Jeddah and Medina are two major cities in the Western Region of Saudi Arabia that constitute approximately 35% of the Saudi population (13).

The inclusion criteria for Riyadh were the same as that for the studies of Jeddah and Medina: all infants born at the study hospitals between January 1, 2010 and December 31, 2011 were included. The prevalence was calculated as the proportion of infants with NSOFC to the total number of births, excluding cases of syndromic orofacial clefting.

The sample size was calculated using Open Source Epidemiologic Statistics for Public Health (OpenEpi) online software (http://www.openepi.com/oe2.3/menu/openepimenu.htm). Factors used in the calculation were the estimated population size (98,000 births/year (14)) and the predicted prevalence based on the mean global prevalence figures (1.25/1000 births) and 95% confidence intervals (CI). This gave a sample size of 61,055 infants to measure the prevalence of NSOFC in Saudi Arabia. We screened 40,005 infants from three hospitals in Riyadh and, by adding data from the Jeddah and Medina studies, collected data on a total of 114,035 infants. A case-control study design was used to assess the relationship between parental consanguinity and NSOFC. The study group included infants born with NSOFC (133) in Riyadh, Jeddah, and Medina and the control group included 233 unaffected infants matched for sex and location.

This study was approved by the King Abdulaziz University research committee, the Institutional Research Review Board (IRB) of the Ministry of Health, and the military hospital. Consent to participate was given by parents.

Procedure

Infants born with NSOFC were identified and the information was passed to a research coordinator. To ensure optimal enrolment, eligible patients were actively pursued every two weeks

through nursing staff working at the neonatal units or neonatal intensive care units of the respective hospitals.

Data was collected through clinical examinations and parent interviews. In addition, a NSOFC diagnosis was confirmed by reviewing medical records and contacting the infants' pediatricians. The total number of infants born with NSOFC in these hospitals over the study period was retrieved from the statistical records of each of the hospitals for that period. NSOFC prevalence was measured by comparing the number of NSOFC cases to the total number of births at each hospital.

NSOFC phenotypes were classified according to LAHSHAL classification (15), which subdivides cleft lip (CL) according to side (right, left, or bilateral) and complete/incomplete (with/without Simonart's band). These subdivisions were used to classify the extent or severity of clefting of the lip in cases of cleft lip (CL) and cleft lip with or without cleft palate (CL/P) (1).

A questionnaire interview with mothers was conducted to obtain data on family history, parental consanguinity, and type of consanguinity (1^{st} cousins, 1^{st} cousins once removed, second cousins, and other type of relatives). The matched control group was used to measure the effects of consanguinity on NSOFC phenotypes and severity.

Statistical analysis

Data were analyzed using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA). Descriptive statistics, such as frequency and percentage, on the epidemiology of NSOFC were analyzed. The Chi square test was used to test for association between consanguinity and the type of NSOFC and severity of CL/P. The significance level was set at $p < 0.05$. The Odds Ratio (OR) and 95% CI were used to measure the effect of consanguinity on NSOFC risk.

Results

Prevalence of NSOFC in Riyadh

At the 3 hospitals in Riyadh, 43 infants were born with NSOFC between January 1, 2010 and December 31, 2011 out of 40,005 births, giving a prevalence of 1.07/1000 births. The prevalence of CL was 0.32/1000 births, of CLP 0.35/1000 births, and of CP 0.4/1000 births (Table 1). Left incomplete CL was the most common NSOFC sub-phenotype, seen in 6 (22.2%) infants (Table 2).

Overall prevalence of NSOFC in Saudi Arabia

When the data from Jeddah and Medina (11, 12)) were added to that from Riyadh, to give a total of 133 births during the study period, a prevalence of 1.17/1000 births was obtained. The prevalence of CL was 0.47/1000 births, of CLP 0.42/1000 births, and of CP 0.28/1000 births. The prevalence of NSOFC was higher in Medina (1.88/1000 births) than in Jeddah (0.81/1000 births), and Riyadh (1.07/1000 births) (Table 1). Associated anomalies were diagnosed in 22.5% of cases. A family history of birth defects was reported in 42.1% of cases, and a family history of orofacial clefting was reported in 22.5% of cases.

Table 2 shows the distribution of NSOFC sub-phenotype according to sex in all three cities. Of the 70 cases of unilateral CL/P, the prevalence of left sided CL/P (CL 28 cases, 21.1%, and CLP 16 cases, 12%) was higher than that of right sided CL/P (CL 13 cases, 9.8%, and CLP 13 cases, 9.8%). The frequency of bilateral CLP (19 cases, 14.2%) was higher than that of bilateral CL (12 cases, 9%). Out of the 101 CL/P cases; the frequency of incomplete clefting of the lip (58 (57.4%)) was higher than complete clefting of the lip (43 (42.6%)).

The prevalence of NSOFC was higher in males (82 cases, 61.7%) than females (51 cases, 38.3%). On the other hand, the prevalence of CP was higher in females (16 cases, 31.4%) than males (16 cases, 19.5%). There was a statistically significant difference in the distribution of the three

NSOFC phenotypes (CL, CLP, and CP) according to sex (P= 0.035). After Chi square adjustment using Bonferroni correction, CL was significantly higher in males than in females (P<0.05).

Effects of city of birth and consanguinity on NSOFC phenotype severity

The prevalence of parental consanguinity was measured for all infants born with NSOFC. Parental consanguinity information was missing in 10 cases, and these were excluded from the analysis. The prevalence of consanguinity among infants with NSOFC in Saudi Arabia was 65.9% (81 cases): 56.1% in Riyadh (23 cases), 77.4% in Medina (41 cases), and 58.6% in Jeddah (17 cases).

There was no relationship between sex and parental consanguinity (P= 0.559). The prevalence of consanguinity was higher for CP (78.6%) than for CL/P (61.1%); however, the difference was not significant (P= 0.11). In addition, the prevalence of severe CL/P (complete clefting of the lip or a bilateral cleft) was higher in infants with consanguineous parents than that in infants with non-consanguineous parents; however, these differences were not significant (P= 0.12 and P= 0.53 respectively, Table 3).

Consanguinity was more prevalent in infants with NSOFC, including all its phenotypes, compared to controls (Table 4). However, the relationship was only statistically significant for CP (P= 0.047, OR: 2.5, and 95% CI: 1 to 6.46). The highest prevalence for CP was for 1st cousin consanguinity, at 72.7% compared to 58.9% for controls.

Discussion

This study describes the characteristics and prevalence of NSOFC in Riyadh, and overall for the cities of Riyadh, Jeddah, and Medina, in Saudi Arabia. We have also assessed the impact of consanguinity on the pattern and severity of NSOFC in Saudi Arabia. Although the data for Jeddah and Medina have been published previously, this paper combines these data with our dataset for a third major city, Riyadh, allowing a more comprehensive picture of oral cleft prevalence in Saudi Arabia.

The prevalence of NSOFC for Riyadh over the 2-year period of the study was 1.07/1000 live births, which is higher than the 0.3/1000 births reported by Kumar *et al.* (1991) for Riyadh. However, that study was hospital based and was conducted in 1991{Kumar, 1991 #154}. Moreover, the prevalence of NSOFC in Riyadh (1.07/1000 live births) and overall 1.17/1000 live births) was lower than the mean global prevalence (1.25/1000 live births) (1). Jeddah showed the lowest prevalence of all cities, while Medina showed the highest; the prevalence for Medina was also higher than the mean global prevalence. This suggests a genetic and geographical influence on the prevalence of NSOFC between regions of Saudi Arabia related to the fact that NSOFC is a multifactorial birth defect (16). Furthermore, that the prevalence of CL is higher than that of CLP, particularly in Medina, differs from global findings with previous studies reporting a higher prevalence of CLP than of CL (17, 18).

The higher consanguinity in Medina compared to Jeddah (19) suggests that consanguinity might have played a role in NSOFC phenotype and, thus, the higher prevalence of CL. Furthermore, studies in countries with low consanguinity show a higher prevalence of CLP than that of CL(20, 21) (20, 21). The higher prevalence of CL/P in males and CP in females is similar to global findings (1).

In children with unilateral CL/P left-sided clefts were more common than right, This is similar to other studies (9, 22, 23) although there is no clear explanation as to why the left side of the lip is more prone to clefting (1). Nevertheless, Johnston and Brown (1980) raised the possibility that the

right side may have better hemodynamic perfusion as fetal head vessels on the right side leave the aortic arch closer to the heart (24).

The prevalence of bilateral clefts was similar to previous studies, which showed approximately 10% for CL and 30% for CLP (25-27). Understanding the pattern of NSOFC sub-phenotypes could inform future genetic research and establish a more personalized approach towards controlling NSOFC in the future.

The prevalence of associated anomalies in this study (22.5%) was higher than those in previous reports on Middle East countries which ranged from 13 to 18% (28) and the mean global . However, other studies have reported values as high as 63% (6, 29, 30). This could be related to methodological differences, variable diagnoses of associated anomalies, and ascertainment. Rittler et al. 2011 reported that 7.1% of NSOFC cases diagnosed as isolated cleft at birth, were found to be associated with other birth anomalies after one year (31).

Family history of orofacial clefting was reported in 22.5% of cases, similar to previous studies carried out across Middle East countries (32-34).

Parental consanguinity and its relationship to oral clefting was assessed in a systematic review carried out on all case-control papers that reported the effect of parental consanguinity on NSOFC. Although the systematic review suggested a positive relationship, it reported a high level of heterogeneity among the included studies(35). Saudi Arabia has a high rate of consanguineous marriage that varies between regions(36). The prevalence of consanguinity in NSOFC in this study was 65.9%, higher than the prevalence of 54.4% reported by Aljohar et al. (2008) (37)for a hospital-based cross sectional study of craniofacial cases in King Faisal Specialized Hospital and Research Centre in Riyadh. It is also higher than the prevalence reported by El Mouzan et al. (2008)(19) and El-Hazmi et al. (1995) (36)for the general Saudi population (57% and 57.7% respectively). The prevalence of consanguinity in NSOFC infants in Medina (77.4%), Riyadh (56.1%), and Jeddah

(58.6%) seen here are similar to those reported by El Mouzan et al. (2008)(19) at 67.2%, 60%, and 44% respectively. Of all NSOFC infants with consanguineous parents, 44 couples (54.3%) were 1st cousins, similar to El-Hazmi et al. (1995)(36) at 41% for the general population.

The higher prevalence of consanguineous marriages in NSOFC compared to the general population could indicate that it is a risk factor in the etiology of NSOFC; this is supported by previous research.(21, 38) However, to confirm this relationship, we used a case-control study design. The prevalence of parental consanguinity was higher for NSOFC and its sub-phenotypes compared to controls; however, the relationship was only statistically significant for CP, with a doubling of the risk. Sabbagh et al. (2014)(35) in their systematic review reported a higher OR for CP (OR: 1.89 and 95% CI: 1.14 to 3.13) compared to CL/P (OR: 1.56 and 95% CI: 1.18 to 2.07 for CL/P). In addition, in the Alamoudi *et al*. (2014) (12)study conducted in Jeddah, a significant relationship was reported between consanguinity and CP (P= 0.039). This could explain why the prevalence of CP in Riyadh and Medina, where consanguinity was high, was higher than Jeddah. Moreover, the higher prevalence of consanguinity in severe CL/P cases support could indicate that parental consanguinity could influence the pattern and severity of NSOFC. Future research on the prevalence of cleft palate sub-phenotypes and its etiology in each region in Saudi Arabia is recommended.

Additionally, stillbirths were not included in this study, which might have caused some bias(5, 39). However, the impact of stillbirth on prevalence is expected to be low as it accounts for 15.7/1000 births recorded by the Ministry of Health, Saudi Arabia (14).

Larger scale national research that includes the private sector, which provides healthcare to approximately 20% of the Saudi population (40), should be considered to describe NSOFC prevalence in the future. Studies that define the relationship between each NSOFC sub-phenotype and, different environmental and genetic risk factors are recommended.

This study contributes to the World Health Assembly (WHA) recommendation in 2010 that all member states should pay attention to birth defects (including OFC) as a significant contributor to the global burden of disease, both in terms of mortality and morbidity.

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Table 1: Prevalence of NSOFC from Jan 1, 2010 to December 31, 2011 by place of birth and phenotype

City	Total births	CL	CLP	CP	Total NSOFC	NSOFC prevalence/1000 births
Riyadh						
King Saud Medical City	13,252	6	4	6	16	1.2
Riyadh National Guard Hospital	16,926	2	6	9	17	1
King Fahad Medical City	9,827	5	4	1	10	1
Total	40,005	13	14	16	43	
Prevalence/1000 births	**1000**	**0.32**	**0.35**	**0.4**		**1.07**
Jeddah	45,896	16	15	6	37	
Prevalence/1000 births	**1000**	**0.35**	**0.33**	**0.13**		**0.81**
Medina	28134	24	19	10	53	
Prevalence/1000 births	**1000**	**0.85**	**0.67**	**0.36**		**1.88**
Overall births	114,035	53	48	32	133	
Overall prevalence/1000 births	**1000**	**0.47**	**0.42**	**0.28**		**1.17**

NSOFC: non-syndromic orofacial clefting; CL: cleft lip; CLP: cleft lip and palate; CP: cleft palate.

Table 2: Distribution of NSOFC sub-phenotypes by place of birth and sex

		Riyadh			Overall, Saudi Arabia		
Phenotype	**Sub-phenotype**	**Male (%)**	**Female (%)**	**Total (%)**	**Male (%)**	**Female (%)**	**Total (%)**
CL N=53 Male: 40 Female: 13	Right incomplete	1 (3.7)	1 (6.3)	1 (3.7)	7 (8.5)	2 (3.9)	9 (6.8)
	Right complete	0	1 (6.3)	0	2 (2.4)	2 (3.9)	4 (3)
	Left incomplete	6 (22.2)	1 (6.3)	6 (22.2)	13 (15.9)	5 (9.8)	18 (13.5)
	Left complete	1 (3.7)	1 (6.3)	1 (3.7)	9 (11)	1 (2)	10 (7.5)
	Bilateral incomplete	1 (3.7)	0	1 (3.7)	8 (9.8)	2 (3.9)	10 (7.5)
	Bilateral complete	0	0	0	1 (1.2)	1 (2)	2 (1.5)
CLP N=48 Male: 26	Right incomplete	0	0	0	5 (6.1)	6 (11.8)	11 (8.2)
	Right complete	1 (3.7)	1 (6.3)	1 (3.7)	2 (2.4)	0	2 (1.5)

Female: 22	Left incomplete	1 (3.7)	1 (6.3)	1 (3.7)	2 (2.4)	2 (3.9)	4 (3)
	Left complete	1 (3.7)	3 (18.8)	1 (3.7)	3 (3.7)	9 (17.6)	12 (9)
	Bilateral incomplete	1 (3.7)	0	1 (3.7)	4 (4.9)	2 (3.9)	6 (4.5)
	Bilateral complete	4 (14.8)	1 (6.3)	4 (14.8)	10 (12.2)	3 (5.9)	13 (9.8)
CP N=32 **Male: 16** **Female: 16**	CP	10 (37)	6 (37.5)	10 (37)	16 (12)	16 (31.4)	32 (24.1)
	Total	**27 (100)**	**16 (100)**	**27 (100)**	82 (100)	51 (100)	133 (100)

NSOFC: non-syndromic orofacial clefting; CL: cleft lip; CLP: cleft lip and palate; CP: cleft palate.

Table 3: Consanguinity and NSOFC phenotype, sex, and severity

Variables	Consanguinity (%)	Non-consanguinity (%)	Total (%)	P value
Gender				
Male	49 (63.6)	28 (36.4)	77 (100)	0.559
Female	32 (69.6)	14 (30.4)	46 (100)	
Total	81 (65.9)	42 (34.1)	123* (100)	
Type of NSOFC				
CL/P	59 (62.1)	36 (37.9)	95 (100)	0.11
CP	22 (78.6)	6 (21.4)	28 (100)	
Total	81 (65.9)	42 (34.1)	123* (100)	
CL in CL/P				
Complete	33 (70.2)	14 (29.8)	47 (100)	0.12
Incomplete	24 (54.5)	20 (45.5)	44 (100)	
Total	57 (62.6)	34 (34.4)	91 (100)	
CL site in CL/P				
Bilateral	20 (66.7)	10 (33.3)	30 (100)	0.53
Unilateral	39 (60)	26 (40)	46 (100)	
Total	59 (62.1)	36 (37.9)	65 (100)	

NSOFC: non-syndromic orofacial clefting; CL: cleft lip; CL/P: cleft lip with/without cleft palate. *The total number is less than 133 due to 10 cases of missing information

Table 4: Comparison of NSOFC and controls for frequency and type of consanguineous marriage

Consanguinity	NSOFC (%)	CL (%)	CLP (%)	CP (%)	Control (%)
Yes	81 (65.9)	31 (63.3)	28 (60.9)	22 (78.6)	138 (59.2)
No	42 (34.1)	18 (36.7)	18 (39.1)	6 (21.4)	95 (40.8)
Total	123 (100)	49 (100)	46 (100)	28 (100)	233 (100)
P value OR 95% (CI)	0.222	0.6	0.836	0.047† 2.5 (1-6.46)	
Type of consanguinity					
1st cousins	44 (56.4)	16 (53.3)	12 (46.2)	16 (72.8)	76 (58.9)
1st cousins once removed	4 (5.1)	3 (10)	1 (3.8)	0	8 (6.2)
2nd cousins	14 (18)	6 (20)	5 (19.2)	3 (13.6)	18 (14)
Relatives	16 (20.5)	5 (16.7)	8 (30.8)	3 (13.6)	27 (20.9)
Total	78* (100)	30* (100)	26* (100)	22 (100)	129 (100)

NSOFC: non-syndromic orofacial clefting; CL: cleft lip; CLP: cleft lip and palate; CP: cleft palate.

*The total number is low due to missing information †Significant relationship

B7: Environmental risk factors in the aetiology of non-syndromic orofacial clefts in the Western Region of Saudi Arabia

Environmental risk factors in the aetiology of non-syndromic orofacial clefts in the Western Region of Saudi Arabia

Introduction:

Non-syndromic orofacial clefting (NSOFC) is described in the literature as orofacial congenital defects that either occur in isolation, or are associated with one major, or several rare, congenital abnormalities (Tolarova and Cervenka 1998). They show significant ethnic and geographic variation and are the most common craniofacial birth defects in the world (Mossey and Modell, 2012). The aetiology of NSOFCs has not been elucidated although it is considered likely that they are multifactorial birth defects, caused by environmental and genetic factors working alone or in combination (Mossey et al., 2009). Previous research has proposed a number of factors, including; maternal medication use, infections, contact with chemicals/ smoking during the first trimester and consanguinity (Al-Bustan et al., 2002; Al-Sahafi, 2010; Czeizel et al., 1984; Elahi et al., 2009; Leitte and Koifman, 2009; Rittler et al., 2001; Taghavi et al., 2012).

Geographic and ethnic variations have been shown to play a part in influencing the aetiology and prevalence of NSOFC (Mossey et al., 2009). There has been little investigation into these factors in the Middle East. Yet it provides a good location to study environmental interactions for NSOFC because the different geographic environments present a variety of factors that can be explored as possibly influencing NSOFC development. Also, the region has a wide mix of ethnicity, again allowing a variety of factors to be explored. This variation makes Saudi Arabia, the largest country in the Middle East (with over 300,000 births/year), a useful part of the world for studying NSOFC and looking into factors influencing the development of these anomalies. This setting also provided the

opportunity to investigate a number of different factors, unique to the Middle East and not previously investigated, including maternal drinking water supply and different types of tobacco smoking.

One third of Saudi Arabian births are in the Western Region (Ministry of Health, 2008). The cities of Jeddah and Maddina have the highest populations of each area, Makkah and Medina in the Western Region (Department of Statistics and Information, 2013). This study is expected to be the first multi-centre case-control study in Saudi Arabia.

The aim of this study was to investigate environmental risk factors and identify those with a relationship to NSOFC in the Western Region of Saudi Arabia.

Materials and methods:

This study is a multi-hospital based case-control study carried out in Jeddah and Maddina cities. It forms part of a series of studies recording baseline data on the prevalence and aetiology of NSOFC in Saudi Arabia.

Study setting and subjects:

Two of the largest, main cities in the Western Region (Jeddah and Maddina) were included in this research. Jeddah city was divided geographically into five districts with a referral center selected for each district (Ministry of Health, 2008 These were: Alazizia Maternity Hospital; King Fahad Hospital; King Abdulaziz University Hospital; King Abdulaziz Medical City; and King Fahad Armed Hospital/Al Mesadia Maternity Hospital (these two hospitals were considered as one center as they are located in the same district and cases were ombined). In Maddina, a single center, Maddina Maternity and Children Hospital, was selected as it was the main child, maternity and cleft referral center.

The study group comprised 112 infants with non-syndromic orofacial clefting (NSOFC), diagnosed by each hospitals' paediatricians (Tolarova and Cervenka 1998). These were infants born or referred to the designated hospitals (e.g. orthodontic clinics for nasal- alveolar molding or plastic surgical department), age 18 months or less (0-18 months) (Table 1). The control group consisted of 138 infants unaffected by orofacial clefting and born at, or attending the designated hospitals for vaccination. The groups were matched for age, gender, and hospital attendance. Both groups were recruited from January 2010 to January 2012. The control infants were picked up and matched by one researcher (HS).

Method:

Ethical approvals for all centers were granted by the Ministry of Health and the Military hospitals research centers. A WHO questionnaire on events in the three month pregestation and the first trimester periods for congenital anomalies was piloted, modified and validated for the Saudi population with additional relevant questions included. Mothers were interviewed face to face. The following information were collected as yes/no responses:

- pregnancy planning, which was defined according to Mossey et al. (2007), as any lifestyle or behavioral changes undertaken prior to conception and/or in early pregnancy to achieve a favourable pregnancy outcome; maternal illnesses and use of medication and supplements; common cold/flu were recorded when mothers self-reported two or more of the following symptoms- fever, feeling feverish, cough, sore throat, runny or stuffy nose, body aches, chills, and fatigue
- maternal stress; threatened abortion; family problems (adverse interactions or events among family members) and severe morning sickness;

- maternal domestic exposure with questions related to solvents (such as; thinner and acetone), pesticides, incense (a certain type of wood producing a fragrant odor when burned produces a fragrant odor) ;
- maternal main drinking water supply during the pregestation three months and first pregnancy trimester such as; tap water, bottled water, or Zamzam water (containing high mineral concentrations and with alkaline pH. This comes from a specific historical well in Makkah city considered to be holy as it is related to two of the prophets; Ibrahim and Ismail); and
- parental tobacco smoking, Jorak smoking (water-pipe or Hookah) and maternal passive smoking (maternal second hand tobacco exposure at home or work)

In addition, further detail was asked for some data areas:

- parental age (maternal age: <20, 21-24, 25-29, 30-34, >34 years; and paternal age: <25, 26-34, 35-39, 40-49, >49 years), infants neonatal weight, and infants' family socioeconomic status (SES) derived from; parental education (high school and/or more; or less), family monthly income (less than 4000RS (threshold for Saudi Salary, https://online.hrdf.org.sa/FAQ/faq.html),4000 to 10,000, more than 10,000) and description of infant's mother living area (urban vs. rural areas); and
- The number of twin cases and family history of congenital abnormalities, and parental consanguinity;

Infant clinical examinations were carried out by two clinicians experienced in the field of cleft lip and palate, to identify NSOFC and classify cleft type as cleft lip (CL), cleft lip and palate (CLP) and cleft palate (CP).

Statistical analysis:

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Data was analyzed with SPSS version 16 (SPSS Inc., Chicago, IL, USA). Descriptive statistics for the epidemiology of NSOFC were presented with frequency and percentages. Chi square was used to test for significant differences in NSOFC risk between the case group and the control group for each exposure variable. The level of significance was set at $P < 0.05$. For significant P values, odd ratios (OR) and 95% confidence of intervals (CI) were calculated to measure the level of risk. To compare the different types of maternal main drinking water supply and their relationship to NSOFC, chi square tests were adjusted using Bonferroni correction. For quantitative variables, t-test was used to compare means. Stepwise forward unconditional logistic regression analysis was carried out on all variables to identify NSOFC risk factors.

Results:

The study included 112 cases (78 from Jeddah and 34 from Maddina) and 138 controls (100 from Jeddah and 38 from Maddina). The 112 cases (66 (58.9%) born in the same hospitals as the control children) comprised; CL (44), CLP (43) and CP (25) cases. Although children with recognized syndromes where orofacial cleft was a feature were excluded, those with other congenital anomalies were included. Anomalies associated with NSOFC were found in 18 cases (15.9%); craniofacial deformities (n=6), cardiovascular anomalies (n=4), genitourinary anomalies (n=4), non-specific failure to thrive (n=2), Central Anomaly (n=1) and multiple anomalies (n=1). The controls comprised 90 (65.2%) infants born in the same hospitals.

The distribution of cleft phenotype for all 112 case infants by cities is described in Table 1. The overall proportion of CL cases (39.3%) is similar to proportion of CLP (38.4%).

Infant neonatal weight in cases (from 1.5 to 4.5kg, with mean=2.98kg) was similar to control (from 1.4 to 4.1kg, with mean=2.89kg) (P= 0.61). Parental age and SES showed no statistically significant differences between cases and controls (P>0.05) (see Table 2). Only one child in each pair of twins was affected with a cleft. The number of twins was significantly higher in the case group (7/108) compared to the control group (1/138) (P= 0.01, OR=9.5, 95% CI: 1.15-78.4). The twins in

the cases group included one affected infant and one non-affected infant. Although there were higher numbers of cases with a family history of consanguineous marriages and congenital abnormalities in cases compared to controls, the differences were not statistically significant (P= 0.24 and P= 0.74 respectively) (see Table 3).

Table 4 details the distribution of different maternal factors among cases and controls and the risk of having an infant with NSOFC. Other medications and diseases that were not mentioned in the table were rarely reported by mothers. These included; urinary tract infection in the pregestation period (six cases and three controls), urinary tract infection in the 1st trimester (three cases and four controls), diabetes (one control), asthma (one case), contraceptives in pregestation period (eight cases and 13 controls), contraceptives in the 1st trimester (three cases and three controls), cortisone (one control), antifungal medication (one case), hypothyroidism and thyroxin (one control), depression (three cases and three controls) antidepressant (one case and one control).

Maternal use of antibiotics during the pregestation and first trimester periods were higher in cases than controls, but the difference was significant only for the pregestation period (P= 0.021, OR=2.71, 95% CI: 1.11-6.62); anti-emetic medication use in the first trimester (P= 0.005, OR=2.85, 95% CI: 1.3-6) was significantly higher in cases than controls.

Also, maternal illness was reported by mothers in the three months pregestation period significantly more often in cases than controls (P= 0.009, OR=2.19, 95% CI: 1.17-4.08) with common cold in the pregestation period being reported significantly more frequently in mothers of NSOFC children than in control group mothers (P= 0.003, OR=3.2, 95% CI: 1.48-7.58).

For maternal supplement use, significantly higher folic acid (P= 0.04, OR=0.58, 95% CI: 0.34-0.98) and calcium (P= 0.02, OR=0.32, 95% CI: 0.11-0.86) taken during the first trimester showed a protective effect against having an infant with NSOFC but was statistically significant only for calcium supplementation use.

Maternal experience of stress, threatened abortion and family problems were more frequently recorded in NSOFC mothers than mothers in the control group but the differences were not significant (P= 0.08 and P= 0.1 respectively). However, severe morning sickness showed a statistically significant association for risk in cases compared with controls (P= 0.006, OR=3.6, 95% CI: 1.34-9.65)

With regard to domestic chemical exposure, significantly more controls were exposed to incense compared to cases (P= 0.03, OR=0.58, 95% CI: 0.34-0.98). Finally, maternal use of mains water supply in the three months pregestation and 1st trimester periods showed a significant difference between cases and controls (P= 0.01). There was no significant difference between drinking tap and bottled water in cases and controls (P=1). However, Zamzam water showed a significant protective effect compared to other types of drinking water (P= 0.004, OR: 0.16, 95% CI: 0.04-0.6 compared to tap water and P= 0.005, OR: 0.2, 95% CI: 0.06-0.7 compared to bottled water) being the main water supply used significantly more often by the control group (P<0.05).

Maternal smoking was rarely reported in the three-month pregestation and 1st trimester period (five times in cases and four in the controls for the pregestation period and only one case in the 1st trimester period). Therefore, only paternal types of smoking were analyzed. Although there was a higher prevalence of paternal smoking in the case group than the control group, the difference was not significant (P> 0.05). However, when looking at the different types of smoking, paternal Jorak smoking was associated with a statistically significantly increased risk of having an infant with NSOFC (P= 0.004, OR=14.07, 95% CI: 1.55-128.1). Maternal passive smoking was also statistically significantly associated with NSOFC (P= 0.05, OR= 2.05, 95% CI: 1.05-4.01) (see Table 5).

Logistic regression analysis was carried out for all variables and their relationship to NSOFC (Table 6). The stepwise logistic regression identified six significant factors as predictors for NSOFC risk: maternal common cold/flu three months pregestation (P= 0.002, OR=4.63, 95% CI: 1.78-12.02); maternal use of antibiotics in the first trimester (P= 0.047, OR 2.36, 95% CI: 1.01-5.5); having a

severe morning sickness (P= 0.001, OR=6.23, 95% CI: 2.04-19.03); paternal Jorak smoking (P= 0.014, OR=15.1, 95% CI: 1.73-131.6); twin pregnancies (P= 0.034, OR 10.77, 95% CI:1.19-97.5); and maternal exposure to incense in the three month pregestation period (P= 0.006, OR=0.42, 95% CI: 0.22-0.77) as predictor variables for NSOFC. Identification of these six factors was implemented in four steps explaining 68% of the risk. Paternal Jorak smoking was identified as the strongest predictor (OR=15.1).

Discussion:

This case-control study is the first investigation into the environmental risk factors for NSOFC in the Western Region of Saudi Arabia. It also the first, to the best of our knowledge to look into the association between the different type of tobacco smoking and maternal drinking water source and NSOFC.

The proportion of CP (22.3%) in the included sample is similar to the global proportion ranging from 20-25% (Mossey and Modell, 2012). The overall proportions of CL (39.3%) were similar to CLP (38.4%), which differs from the global figures where there is a higher CLP proportion (40-45%) compared to CL (20 to 25%) (Mossey and Modell, 2012). Also, the proportion of the different phenotypes of NSOFC in Jeddah differed from that of Maddina. These variations could be due to different hospital facilities resulting in more infants with CLP than CL being referred to Jeddah Hospitals where there are more established treatment facilities. However, it could also indicate the need for studies that detect specific environmental risk factors for different geographic areas. These kinds of studies have an important role in disease prevention (Mossey and Castilla, 2001).

Previous studies have suggested a significant relationship between parental SES and having a child with NSOFC and have related their finding to the parental healthcare and life style (Taghavi et al., 2012). Others have reported a higher risk of NSOFC in rural areas compared to urban (Messer et al., 2010; Al-Sahafi, 2010). However, our study found no significant relationship between SES and

NSOFC and this might be related to the general higher SES of Saudis compared to other communities, availability of free governmental healthcare and generally high living standards.

In common with other studies (Jamilian, 2007; Kanaan, 2008; Al-Sahafi, 2010), we found no association with parental age and NSOFC. In 2012, a meta-analysis carried out to assess the relationship between parental age and NSOFC found heterogeneity between studies looking at parental age and their relationship to cleft lip with or without cleft palate (CL±P). A relationship was only noted between parental age and cleft palate only (Herkrath et al., 2012). Neonatal weight was similar in both case and control groups, confirming previous findings (Leitte and Koifman, 2009; Welch and Hunter, 1980), although Bonaiti et al (1982), in their epidemiological study set in France, reported lower birth weight for CP (Bonaiti et al., 1982).

Congenital anomalies have been reported to be higher in twins compared to singletons (Glinianaia et al., 2008). Although the number of twins in this study sample was low, there was a significantly higher number of twins in the study group compared to controls (P= 0.04). However, in 2012, Danish national research found no increased risk of NSOFC in twins compared to singletons although they did note that the recurrence risk for clefts was greater in twins than in non-twin siblings and that heritability estimates were over 90% (Grosen et al., 2012).

Maternal illnesses and medication use in the three-month pregestation period were significantly related to having an infant with NSOFC. However, this relationship was not significant for maternal illness in the first trimester. Mothers may not be aware of their pregnancy until the end of the second gestation month, which is a critical time for craniofacial development. Also, the three months pregestation period covers the time around conception which could affect the embryonic development (Mossey and Castilla, 2001). The positive relationship between maternal illness and having a child with NSOFC was supported by Hashmi et al (2010) study who reported a protective effect with maternal antipyretic medication use. This study also found a higher number of mothers taking antipyretics in the control group than in the NSOFC group although there was no

statistically significant differences (P>0.05). The effect of antipyretic medications could explain the relationship between maternal illness and NSOFC. It could suggest that the disease symptoms, possibly pyrexia, lie behind this relationship.

Our finding that maternal use of antibiotics increased risk of NSOFC (P= 0.01, OR=3) is supported by another study (Lin et al., 2012). In a nationwide cohort study of 806,011 live births in Denmark it was concluded that although maternal antibiotic use was not a major risk factor for NSOFC, there was a significant relationship between certain classifications of antibiotics (tetracyclin, sulfamethizole, doxycycline, trimethoprim) and NSOFC (Mølgaard-Nielsen and Hviid, 2012).

Maternal supplement use during the first trimester showed a protective effect against having a child with NSOFC for both folic acid and calcium supplement use (P= 0.04, OR= 0.58, 95% CI: 0.34-0.98 and P= 0.02, OR=0.32, 95% CI: 0.11-0.88 respectively). For folic acid, the finding is supported by a meta-analysis carried out by Bodovinac et al (2007), including five prospective studies, where a significant relationship was identified between folic acid and NSOFC occurrence (OR=0.55, 95% CI: 0.32-0.95) (Badovinac, et al., 2007). Like this study, Jia et al (2011) noted a significantly protective effect with calcium supplement use for CL±P (0.02, OR= 0.66, 95% CI: 0.47-0.93). This relationship could be related to the severe Vitamin D deficiency level in Saudi women ; one of the highest Vitamin D deficiency level in the world (Elshafie et al., 2012). Vitamin D is known to play an important role in the maintenance of serum (ionised) calcium levels (Nordin, 2010).

Smoking was more frequent in NSOFC group than the control group but the differences were not significant for all types of smoking. Passive smoking and Jorak had a statistically significant relationship with NSOFC (P= 0.05 and P= 0.004 respectively). Previous researchers have reported a positive significant relationship between smoking or passive smoking and having an infant with NSOFC (Jia et al., 2011; Little et al., 2004; Zhang et al., 2011). However, this is the first study looking at the type of smoking device. Previous research has found that Jorak smokers have significantly higher carboxyhaemoglobin concentration than cigaratte smokers, higher carbon monoxide and

have higher serum cotinine levels due to the larger amount of tobacco consumed during water-pipe smoking compared with cigarette smoking (Ardawi et al., 2007; Zahran et al., 1985). Water-pipe smoking has recently been indicated to be an emerging global epidemic concern and is largely understudied (Fakhreddine et al. 2014). This paper provides further evidence and supports the need to study the association between different smoking exposure devices and mechanisms including water-pipe and passive smoking in relation to congenital anomalies.

The positive significant relationship between NSOFC infants and mothers experiencing severe morning sickness or use of anti-emetic medication is supported by Miller (2002), who suggested that severe morning sickness can lead to loss of around 5% of maternal original weight, and could result in birth defects. Also, Jia et al (2011) found a significant protective effect for maternal weight gain during pregnancy and having a cleft child (OR = 0.15, 95% CI: 0.034–0.63). However, this is not a clear picture as other studies suggest protective effects or no relationship between NSOFC and maternal nausea and vomiting (Czeizel et al., 2002; Molina-Solana et al., 2013; Czeizel et al., 1984; Molina-Solana et al., 2013; Badovinac et al., 2007).

To the best of our knowledge, this research is the first to find associations in two areas, between the occurrence of NSOFC and 1) maternal use of incense and 2) source of maternal drinking water. These are interesting preliminary observations that, if confirmed in future studies, could raise the possibility for community prevention programs through water supplication and air incense exposure in different closed areas. The relationship between maternal water supply and NSOFC could be related to the concentration of mineral water content with a greater mineral content in Zamzam water compared to tap and bottled water. It contains the highest amount of minerals such as: calcium, fluoride, zinc and magnesium (Al Zuhair and Khounganian; Shomar, 2012; Zamzam Studies and Research Centre, 2011; Alfadul and Khan, 2011). Two of these minerals (zinc and calcium) have been previously found to have a significant relationship with NSOFC (Hozyasz et al., 2009; Jia et al., 2011; Krapels et al., 2004; Munger et al., 2009; Tamura and Goldenberg , 1996).

The protective effect found for maternal incense exposure (P= 0.03, OR=0.58, 95% CI: 0.34-0.98) could be related to the putative antibacterial effects on the surrounding air of burned incense (Bevilacqua et al., 1997; Twort and Baker, 1940). In addition, a Hong Kong study looking at the confounding effect of the association between air pollution and female lung cancer found that incense had no effect on lung cancer risk among nonsmokers and significantly reduced the risk of cancer in smokers (P= 0.01) (Koo and Ho, 1996).

As well as the inherent bias of recall bias found in all case-control studies of this nature, one of its limitations was that some of the significant environmental factor exposures were found in five or less mothers, reducing the reliability of the statistical result (twins, paternal Jorak smoking and maternal drinking water). However, these were still mentioned to complete the data picture. Also, this study assessed NSOFC as a single group and because of the small sample size we were not able to subdivide them according to their sub-phenotypes (cleft lip with or without cleft palate (CL/P) and CP). However, future research with larger sample sizes that assesses the risk factors for CL/P and CP individually is recommended.

Finally, there is a confounding factor effect that might bias the results. In order to overcome this limitation, logistic analysis was carried out. Variables that were found to be significant with logistic regression analysis and increased NSOFC risk included; maternal common cold/flu three months pregestation, maternal use of antibiotics in the first trimester, having a severe morning sickness, paternal Jorak use and, and twin pregnancies. On the other hand, maternal exposure to incense in the three-month pregestation period significantly decreased NSOFC risk in logistic regression analysis. Variables which were excluded by the logistic regression analysis might be considered to be confounding factors in the aetiology of NSOFC.

Conclusion:

In this Middle Eastern population, maternal use of antibiotics, maternal common cold/flu, paternal Jorak smoking and maternal passive smoking and twin births were found to be risk factors for NSOFC. Folic acid and calcium supplement use had a tendency to decrease the risk of having an infant with NSOFC. In addition, incense and Zamzam water showed a protective effect against having an infant with NSOFC. These findings, if confirmed in future studies, could raise the possibility of introducing community preventive programs through changing water mineral content and recommending use of incense. Further studies are also required to verify this and to identify the potential role of gene—environment interaction, and the interaction between different environmental risk factors in primary prevention.

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Table 1: Distribution of cleft phenotype by city for all case infants aged 18 months or less (n=112), for the two year recruitment period.

City	CL (%)	CLP (%)	CP (%)	Total
Jeddah	29 (37.2)	34 (43.6)	15 (19.2)	78 (100)
Maddina	15 (44.1)	9 (26.5)	10 (29.4)	34 (100)
Total	44 (39.3)	43 (38.4)	25 (22.3)	112 (100)

Table 2: Distribution of case infants and control infants according to parental age and socioeconomic status. Responses were not received for all questions and response numbers are given.

Variables	Cases/ total (%)	Controls/total (%)	P value
Parental Age (cases=104 [a], controls=138)			
Maternal age			
<20	6 (5.8)	3 (2.2)	0.419
20-24	18 (17.3)	26 (18.8)	
25-29	28 (26.9)	48 (34.8)	
30-34	31 (29.8)	34 (24.6)	
>34	21 (20.2)	27 (19.6)	
Paternal age			
<25	6 (5.8)	6 (4.3)	0.425
25-39	69 (66.3)	101 (73.2)	
40-49	23 (22.1)	28 (20.3)	
>49	6 (5.8)	3 (2.2)	
Socioeconomic status			
Parental education (Less than high school)			
Paternal education	28/104[a] (26.9)	45/138 (32.3)	0.34
Maternal education	27/103[a] (27.3)	51/138 (37)	0.08
Family monthly income in Saudi Ryal (RS) (cases=104 [a], controls=137[a])			
Less than 4000 (RS)	26 (25)	38 (27.7)	0.2
4000 to 10,000 (RS)	64 (61.5)	90 (65.7)	
More than 10,000 (RS)	14 (13.5)	9 (6.6)	
Description of living place (Rural)	17/103[a] (16.5)	17/136[a] (12.5)	0.38

*Statistically significant differences P≤0.05.

[a] number was less than the total due to missing information

Table 3: Distribution of case infants and control infants according to number of twin cases, pregnancy planning and family history variables; odds ratio adjusted for maternal age

Variables and risk factor	**Cases/ total (%)**	**Controls/total (%)**	**P value** **OR 95 % (CI)**
Number of cases with twins	7/108[a,c] (6.2)	1/138[a] (0.7)	**0.01*** 9.5 (1.15-78.4)
Pregnancy planning	39/104[a] (37.5)	54/138 (39.1)	0.8 0.93 (0.55-1.58)
Family history			
Infants with positive family history for congenital abnormalities	45/101[a] (44.6)	50/137[a] (38)	0.24 1.2 (0.72-2.08)
Parental consanguinity	59/103[a] (57.3)	75/136[a] (55.1)	0.74 1.04 (0.62-1.77)
Type of parental consanguinity:			
1st cousins	39 (66.1)	52 (70.3)	0.83
2nd cousins	11 (16.8)	12 (14.9)	
Relatives	9 (15.3)	11 (14.9)	
Total	59 (100)	75 (100)	

*Statistically significant differences P≤0.05

[a] number was less than the total due to missing information

[c] the included twins had one affected infant and one no-affected infant (non-cleft infant).

Table 4: Distribution of cases and controls according to different maternal factors and their risk factors with odds ratio adjusted for maternal age.

Variables and risk factors	Cases/ total (%)	Controls/total (%)	P value OR 95 % (CI)
Maternal medication use and illness:			
Antibiotic use pregestation	16/102[a] (15.7)	8/138 (5.8)	**0.021*** 2.71 (1.11-6.62)
Antibiotic use 1st trimester	18/103[a] (17.5)	13/138 (9.4)	0.058 2.09 (0.96-4.53)
Antipyretic medication pregestation	16/104[a] (15.4)	26/138 (18.8)	0.58 0.82 (0.4-1.64)
Antipyretic medication 1st trimester	18/104[a] (17.3)	30/138 (21.7)	0.39 0.76 (0.4-1.43)
Anti-emetic medication first trimester	22/103[a] (21.4)	12/138 (8.7)	**0.005*** 2.85 (1.3-6)
Illness pregestation	31/103[a] (30.1)	22/138 (16.1)	**0.009*** 2.19 (1.17-4.08)
Illness 1st trimester	38/103[a] (36.9)	48/138 (35.8)	0.87 1.03 (0.59-1.78)
Common cold/flu pregestation	20/103[a] (19.4)	9/136[a] (6.6)	**0.003*** 3.32 (1.48-7.58)
Common cold/flu 1st trimester	22/103[a] (21.4)	26/136[a] (19.1)	0.67 1.09 (0.57-2.08)
Fever pregestation	13/10[2a] (12.7)	9/136[a] (6.6)	0.11 2.08 (0.85-5.08)
Fever 1st trimester	13/103[a] (12.6)	18/136[a] (13.2)	0.89

			0.93 (0.43-1.99)
Maternal supplements use			
Folic acid pregestation	8/96[a] (7.7)	10/128 (7.2)	0.91 1.06 (0.4-2.81)
Folic acid 1st trimester	60/104[a] (57.7)	97/138 (70.3)	0.043 0.58 (0.34-0.98)
Multivitamins pregestation	6/104[a] (5.8)	5/138 (3.6)	0.44 1.84 (0.53-6.34)
Multivitamins 1st trimester	16/103[a] (15.5)	17/137[a] (12.4)	0.49 1.22 (0.58-3.96)
Iron pregestation	8/103[a] (7.8)	8/138 (5.8)	0.54 1.43 (0.51-3.96)
Iron 1st trimester	20/103[a] (19.4)	40/138 (29)	0.09 0.62 (0.33-1.15)
Calcium 1st trimester	5/104[a] (4.8)	19/138 (13.8)	**0.02*** 0.32 (0.11-0.86)
Maternal stress			
Threatened abortion	7/102[a] (6.9)	3/136[a] (2.2)	0.08[C]
Severe morning sickness	15/102[a] (14.7)	6/136[a] (4.4)	**0.006*** 3.6 (1.34-9.65)
Family problem	33/99[a] (33.3)	32/136[a] (23.5)	0.1 1.7 (0.96-3.05)
Maternal domestic environmental exposure			
Maternal exposure to solvent pregestation	19/102[a] (18.6)	22/138 (15.9)	0.59 1.16 (0.59-2.29)
Maternal exposure to solvent 1st	14/102[a] (13.7)	21/138 (15.2)	0.9

trimester			0.89 (0.4-1.83)
Maternal exposure to pesticides pregestation	23/102[a] (22.5)	30/138 (20.7)	0.43 1.08 (0.58-2)
Maternal exposure to pesticides 1st trimester	25/102[a] (24.5)	32/138 (23.2)	0.81 1.09 (0.6-2)
Maternal exposure to incense pregestation	54/102 (52.9)	91/138 (65.9)	0.053 0.6 (0.36-1.02)
Maternal exposure to incense in the 1st trimester	53/102[a] (52)	91/138 (66)	**0.03*** 0.58 (0.34-0.98)
Maternal main drinking water type			
Tap water	28 (28.6)	29 (21.5)	**0.01***
Bottled water	67 (68.4)	87 (64.4)	
Zamzam water	3 (3.1)	19 (14.1)	

*Statistically significant differences P≤0.05

[a] number was less than the total due to missing information

[c] OR can't be adjusted because one of the table numbers was zero

Table 5: Distribution of cases and controls according to different forms of smoking with odds ratio adjusted for maternal age.

Variables and risk factors	**Cases/ total (%)**	**Controls/total (%)**	**P value** **OR 95 % (CI)**
Paternal smoking	37/102[a] (36.3)	42/137[a] (30.7)	0.36 1.73 (0.7-2.37)
Paternal tobacco smoking	26/102[a] (25.5)	35/138 (25.4)	0.98 1.03 (0.57-1.86)
Paternal Jorak smoking	8/102[a] (7.8)	1/138 (0.7)	**0.004*** 14.07 (1.55-128.1)
Maternal passive smoking	25/102[a] (24.5)	21/137[a] (15.3)	**0.05*** 2.05 (1.05-4.01)

*Statistically significant differences P≤0.05

[a] number was less than the total due to missing information

Table 6: Multiple logistic regression analysis for all variables showing the most significant factors related to NSOFC with a cutoff value of 0.5, probability for stepwise entry 5% and probability for stepwise exclusion 10%.

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Factors	P value	OR and 95% (CI)
Factors associated with increased risk of NSOFC		
Twin pregnancies	**0.03***	10.79 (1.9-97.5)
Common cold/flu pregestation	**0.002***	4.63 (1.78-12.02)
Maternal antibiotic use in the 1st trimester	**0.047***	2.36 (1.01-5.5)
Severe morning sickness	**0.001***	6.23(2.04-19.03)
Paternal Jorak smoking	**0.014***	15.1 (1.73-131.6)
Factors associated with decreased risk of NSOFC:		
Incense pregestation	**0.006***	0.42 (0.22-0.77)

*Statistically significant differences $P \leq 0.05$