IRF6 GENE VARIANT AND ENVIRONMENTAL RISK FACTORS IN NON-SYNDROMIC CLEFTS OF THE LIP AND/OR PALATE IN A KENYAN POPULATION

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DECLARATION

I ...EMILY NYAMU..... declare that this thesis entitled "IRF6 gene and environmental risk factors in non-syndromic clefts of the lip and/or palate in a Kenyan population" is the result of my work and has not been submitted for the award of a degree in this or any other university.

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DEDICATION

This thesis is dedicated to my family for their loving support and encouragement.

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DEFINITION OF TERMS

Allele

An alternative form of a gene (one member of a pair) that is located at a specific position on a specific chromosome.

Delta Normalized Reporter (ΔRn)

The magnitude of the signal generated by a set of PCR conditions.

Karyotype

The characteristic chromosome complement of a eukaryote species

Phenotype

Any observable characteristic or trait of an organism: such as its morphology, development, biochemical or physiological properties, or behavior, as determined by both genetic makeup and environmental influence.

Threshold cycle (C_T)

Cycle number at which the fluorescence intensity exceeds the threshold intensity.

ABBREVIATIONS

A	Adenosine
C	Cytosine
CL	Cleft lip
CLP	Cleft lip and palate
CL+/-P	Cleft lip with or without cleft palate.
CP	Cleft palate
C_T	Threshold cycle
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
G	Guanine
IRF6	Interferon Regulatory Factor 6
KEMRI	Kenya Medical Research Institute
MDS	Master of Dental Surgery
MSX1	Drosophila msh homeo box homolog-1
MTHFR	5,10-Methylenetetrahydrofolate reductase
OMFS	Oral and Maxillofacial Surgery
PCR	Polymerase Chain Reaction
PVRL1	Poliovirus receptor like-1
RFC1	Reductase folate carrier-1
SNP	Single nucleotide polymorphisms
SPSS	Statistical Package for Social Sciences
T	Thymine
TBX22	T box transcription factor-22
TE	Tris buffer with ethylenediaminetetraacetic acid
TGFA	Transforming growth factor-alpha
TGFB3	Transforming growth factor beta-3
Tris- HCL	Tris buffer with hydrochloric acid
UoN	University of Nairobi
VDW	Van der Woude syndrome
ΔRn	Delta Normalized Reporter

ABSTRACT

Background: Non-syndromic cleft lip and/or palate are common craniofacial congenital disorders. Their aetiology is largely unknown but genetic and environmental factors have been implicated. In this regard, various genes have been studied as the causative factors. One such gene is the Interferon Regulatory Factor 6 (IRF6) gene whose mutations have been found to cause the Van der Woude syndrome, a disorder characterised by lower lip pits with or without cleft lip/palate. Unlike the other genes that have been studied so far, the IRF6 gene shows consistency in the association of its mutations to non-syndromic cleft lip with or without cleft palate (CL+/-P) among various populations. There is a paucity of information regarding the contribution of these gene variations to non-syndromic CL+/- P in the indigenous African population.

Objective: To determine the presence of an IRF6 gene variant in non-syndromic cases of CL+/- P in an indigenous African population.

Material and Method: A descriptive cross-sectional study was conducted utilizing a purposive sampling method to select hospitals, in the urban and rural areas, where the Operation Smile and Smile Train Missions were offering free treatment for CL+/- P and cleft palate (CP) patients. Convenient sampling was used to recruit 113 patients with non-syndromic orofacial clefts with their accompanying biological parents. DNA was extracted from buccal cell samples and the presence of an IRF6 gene variant (rs2013162) was determined. Environmental risk factors were determined from a questionnaire.

Data Analysis: Frequency distributions and proportions were used for the description of findings. The Chi square with Yates correction and Wilcoxon

Signed Rank's tests were used to determine the relationship between the variables.

Results: Of the 113 children in the study, 65 were male while 48 were female. The age of presentation ranged between 1 month and 15 years with a mean of 26 months. Majority of the children (87.6%) were in the 0-5-year age group. At the birth of a cleft afflicted child, most of the mothers (69%) were below the age of 30 years while most of the fathers (54.9%) were above 30 years of age. The pattern of clefts was 53.1% for CL, 43.4% for CLP and 3.5% for CP. A female predilection was observed for the CL and CP phenotypes while a male predilection was noted for the CLP phenotype. The IRF6 gene variant (rs2013162) was present in 3 of the children and 3 of the mothers studied. Only one of these mothers transmitted the gene variant to her child. None of these children was exposed to any of the other risk factors studied. Only 1 mother gave a history of smoking and 4 of alcohol use during pregnancy. Almost a quarter of the mothers had used prescription drugs during pregnancy and familial clefts were reported in 14% of the families.

Conclusion: The IRF6 gene variant was present to a limited extent in the Kenyan population studied (2.7% of the children and 2.7% of the mothers). This suggests that perhaps a different IRF6 gene variant or a different gene altogether may contribute to non-syndromic clefts in the African population studied. It is also possible that the non-syndromic clefts in the African population have a higher environmental as opposed to genetic influence in their aetiology. However, a large population study is recommended to ascertain these implications. Increasing paternal age and prescription drug history during pregnancy

(antimalarials and antibiotics) may be important risk factors to clefting in the population studied.

CHAPTER ONE

1.0. INTRODUCTION AND LITERATURE REVIEW

Epidemiology

Cleft lip (CL), cleft palate (CP) or a combination of the two (CLP), are the most common craniofacial congenital malformations (Stainer and Moore, 2004). They are the second commonest congenital malformations in the body, exceeded only by cardiovascular anomalies (Wantia and Rettinger, 2002). On average, these orofacial clefts have a birth prevalence rate ranging from 1/1000 to 2.69/1000 amongst populations in different parts of the world (Mc Leod et al. 2004). A distinct ethnic variation is seen in orofacial clefts. The rates of oral clefts in Asian populations are high (0.79 to 3.74 per 1000 individuals) while intermediate rates for Caucasians have been reported (0.91 to 2.69 per 1000 individuals); and Black African populations appear to have a very low rate of cleft malformation (0.18 to 1.67 per 1000 individuals) (Wantia and Rettinger, 2002).

In a study in the US comparing prevalence rates of different cultures to countries of origin, it was reported that African Americans had a lower prevalence rate of CL +/- P when compared to Caucasians. A prevalence rate of 0.61 per 1,000 and 1.05 per 1,000 live births respectively was reported by Croen et al. (1998). In Malawi there is a reported low prevalence rate for CL+/- P of 0.7 per 1,000 live births Msamati et al. (2000). Suleiman et al. (2005) found that the prevalence rate of clefting among a group of Sudanese hospital new-borns in Khartoum was 0.9 per 1,000 live births. Similarly, a low prevalence rate of 0.17 per 1,000 live births has been reported in a Tanzanian hospital based study by Beston and Fabian, (2007).

Reported data on the prevalence of orofacial clefts vary according to the investigator, methodology and the region of study. Although the total combined frequency of CL, CLP and CP is often used in statistics, it is necessary to realize that combining the two aetiologically different groups (CL+/- P and isolated CP) represents a misclassification bias. It is also necessary to distinguish between syndromic and non-syndromic cases for the same reason. Another reason for the varied prevalence is that some studies include all pregnancies (live births as well as pregnancy loses). There is evidence to suggest that the development of clefts in stillbirths and abortions is three times more frequent than in live births (Wantia and Rettinger, 2002). Therefore, this distinction should be made when reporting prevalence.

The pattern of distribution of orofacial clefts seems to show an ethnic variation as well. In the Caucasians, the pattern of distribution is consistent with CLP accounting for 50% of the cases, CL accounting for 25% and CP occurring in 25% of the cases (Regezzi et al. 2008). Tan et al. (2008) reported a varied pattern of distribution in the Asian population. In that study, CLP accounted for 48.7% of the cases, CL accounted for 19.1% of the cases and CP accounted for 32.2% of the cases. Similarly, a study of the Arab population by Aljohar et al. (2008) reported CLP to have accounted for 48% of the cases, CL for 15% of the cases and CP for 36% of the cases. The Arab and Asian populations seem to have higher proportions of the CP variant. In the African population a departure from the proportions seen in the Caucasian, Arab and Asian populations was noted. A study by Osundwa (2005) reported these proportions as 47% for CLP, 46% for CL and 7% for CP in a Kenyan population. Onyango and Noah, (2005)

also reported similar proportions in a Kenyan population with CLP accounting for 50% of the cases, CL for 44% of the cases and CP for 6% of the cases. Wachira, (2009) in a Kenyan study with a retrospective and prospective arm reported CLP to have been more prevalent in the retrospective arm and CL to have been more prevalent in the prospective arm. This may indicate a change of trend in the pattern of distribution of clefts within the Kenyan population. A Nigerian study reported higher proportions of the CLP phenotype (Orkar et al. 2002). Diverse African populations seem to have a lower proportion of the CP variant. The variations in the pattern of distribution of clefts may be racial or due to different environmental exposures based on geographical distribution.

Regarding the distribution of clefts with respect to gender, the following sex ratios have been reported: a large study of 8,952 orofacial clefts in whites found the male to female ratio to have been 1.50-1.59:1 for CL, 1.98-2.07:1 for CLP, and 0.72-0.74:1 for CP (Tolarova, 1990). The Asian study by Tan et al. (2008) reported a male to female ratio of 1:1 for CL, 1.3:1 for CLP and 0.6:1 for CP. Osundwa, (2005) in a study of a Kenyan population reported a male to female ratio of 1.12:1 for CL, 1.17:1 for CLP and 2.89:1 for CP. Another Kenyan study by Onyango and Noah, (2005) showed a similar trend with the male to female ratio for CL reported as 1.5:1, CLP as 1.1:1 and CP as 1.1:1. Kenyan studies seem to show a male predilection for all cleft types. Usually males are more often affected with CL+/- P, whereas females are more frequently afflicted with CP only and with severe forms of CL+/- P (Wantia and Rettinger, 2002). The reason for this pattern of distribution based on gender is not known (Shapira et al. 1999). However, it has been postulated that gender differences in the incidence of CP may be

related to differences in the timing of palate development. The palatal shelves move horizontally and begin to fuse by the seventh week in a male foetus. This does not occur until eight and a half weeks in a female foetus, providing a longer window of vulnerability to teratogens (Yetter, 2002).

Classification

The group of orofacial cleft anomalies is heterogeneous and comprises of typical orofacial clefts (CL, CP, CLP) and atypical clefts including the median, transverse, oblique and other types of facial clefts (Tolarova and Cervenka, 1998, Tessier, 1976). Typical and atypical clefts can occur as part of a syndrome but more commonly occur in isolation (Blanton et al. 2005). Non-syndromic cases are the majority and account for over 70% of the cases (Wantia and Rettinger, 2002, Zucchero et al. 2004, Scapoli et al. 2005). The syndromic cases are accounted for by several hundred mendelian, non-mendelian, chromosomal, teratogenic and sporadic conditions that include other birth defects (Zucchero et al. 2004). Children with associated anomalies are more likely to have combined CLP or isolated CP as opposed to CL (Millerad et al. 1997). They are often of low birth weight as well. The non-syndromic forms of orofacial clefts on the other hand, are likely due to gene–environment interactions (Ghassibe et al. 2005).

A classification system for orofacial clefts that is universally accepted has been sought after and many models are now available of varying complexity. A good classification of cleft anomalies is fundamental for treatment, for genetic and aetiopathological studies and for preventive measures correctly targeting the category of preventable orofacial clefts. Such a system would be one that tells

more about a cleft than simply the broad category it fits in but one that does not have too much detail that the more complex systems have. Some of the more accepted classifications include:

- Davies and Ritchie classification (Davies and Ritchie, 1922).
- The Veau classification system (Millard, 1994).
- Fogh-Andersen classification (Fogh-Andersen, 1971).
- Kernahan and Stark classification (Wayne et al. 1998)
- International Confederation of Plastic and Reconstructive Surgery Classification (Adopted following the Rome Congress of the International Confederation of Plastic and Reconstructive Surgery in 1967).
- LAHSAL classification (Kriens, 1989).
- World Health Organization International Statistical Classification of Diseases and Related Health Problems 10th Revision, Version for 2007*

Embryology and Pathogenesis of clefting

In the fourth week of intra-uterine life, neural crest cells proliferate rapidly and migrate into the face and neck region to form the five facial primordia that appear as prominences around the stomodeum (primitive oral opening). These prominences include:

- The single frontonasal prominence
- Paired maxillary prominences
- Paired mandibular prominences.

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* Classification systems are indexed (Appendix 1).

The frontonasal prominence forms the forehead, nose, and the top of the primitive mouth. The maxillary prominences form the lateral sides of this structure and the mandibular prominences make up the caudal boundaries (Bender, 2000, Moore and Persaud, 2003). During the fifth to eighth week, the maxillary processes on each side of the mouth grow forward and fuse with the lower edges of the lateral nasal process. They extend below the nasal pits to reach and merge with the medial nasal process which forms the philtrum of the upper lip and primary palate. This produces a continuous ridge above the mouth that forms the upper lip and the primary palate up to the incisive foramen (Moore and Persaud, 2003, Thigpen and Kenner, 2003).

Mesodermal tissue migrates from the first branchial arch and reinforces the fused tissues in the developing lip. This tissue assumes a medial position and the two masses formed will assume a lateral position. If this process is delayed, or if one mass is absent, the branchial membrane will pull apart and a CL will develop. Failure of merging between the medial nasal and maxillary processes on one or both sides results in a unilateral or bilateral CL. Median CL occurs due to failure of the medial nasal prominences to merge. The cleft may affect only the upper lip or it may extend more deeply to involve the primary palate (CL and alveolus). If the fusion of palatal shelves is also impaired, the CL is accompanied by CP forming the CLP abnormality (Wantia and Rettinger, 2002). The CP in this case occurs as a secondary event due to the abnormal position of the tongue caused by cleft of the lip and primary palate (Wantia and Rettinger, 2002).

The secondary palate develops from the right and left palatal processes. Fusion of palatal shelves begins at the 8th week of the foetal period and continues usually until the 12th week. The lateral palatine shelves grow from the lateral walls of the primitive mouth. Initially the palatal shelves lie vertically under the tongue. Development of the jaw results in a relatively smaller tongue, which moves inferiorly, allowing the palatal shelves to grow toward each other and elevate to a horizontal position. The palatal shelves fuse with the nasal septum and primary palate. They first meet and fuse in the midline in the posterior part, where they merge with the nasal septum (Wantia and Rettinger, 2002, Bender, 2000, Moore and Persaud, 2003, Thigpen and Kenner, 2003). By 12 weeks, the fusion is complete and bone extends from the maxillae and palatine bones into the palatal shelves forming the hard palate. The part that fuses but does not ossify or contact the nasal septum forms the soft palate and uvula (Moore and Persaud, 2003, Thigpen and Kenner, 2003). A CP occurs when this fusion fails. One hypothesis is that a threshold exists beyond which delayed movement of palatal shelves does not allow closure to take place and this results in a CP which is a partial or total lack of fusion of the palatal shelves. It can, therefore, occur due to:

- Defective growth of palatal shelves
- Failure of the shelves to attain a horizontal position
- Lack of contact between shelves
- Rupture after fusion of the shelves

- Failure of the epithelium covering the processes to undergo apoptosis, to allow fusion to take place.

From the embryology, it is apparent that CL+/- P and isolated CP are different entities with different embryological origins.

Aetiology and Risk Factors

The aetiology of orofacial clefts is difficult to understand because normal orofacial development is a complex and highly regulated process that involves many signalling pathways. Hence, these disorders are postulated to result from multiple factors, both genetic and environmental, interacting together during a critical stage of development (Murray and Schutte, 2004). They may be caused by a genetic derangement leading to a malformation, the influence of teratogens leading to a disruption of a normal developmental process, or mechanical forces that interfere with normal tissue formation that results in a deformation (Thigpen and Kenner, 2003).

The role of environmental factors in the aetiology of CLP is unclear, with some studies identifying numerous environmental risk factors and others showing little evidence of the association of environmental risk factors to isolated non-syndromic oral clefts (Wyszynski and Beaty, 1996, Beaty et al. 2001, Christensen et al. 1995). The following are environmental risk factors that have shown evidence of association with orofacial clefting.

- Maternal age of less than 19 years (De Roo et al. 2003), or more than 39 years (Shaw et al. 1991). This increased susceptibility of clefting is only noted when CL+/- P is considered as a group as opposed to when CL and CP are considered separately (Carinci et al. 2003).

- Increasing paternal age causes a small but significant increase in the incidence of clefting (Thigpen and Kenner, 2003).

- Teratogens may contribute to CL+/- P by disrupting a normal developmental process at a critical stage of development. Several medications have been linked to the development of CL+/- P when taken during the first trimester, a critical period of development for the lips and palate. These include benzodiazepines, phenytoin, opiates, penicillin, salicylates, cortisone and high doses of vitamin A (Bender, 2000, Thigpen and Kenner, 2003, Carinci et al. 2003, Diewert and Pratt, 1981, Melnick et al. 1981). Monotherapy is recommended for pregnant patients on anticonvulsants and folate therapy is particularly important for these women (Pennel, 2003, Murray, 2002).

- Smoking during the first trimester increases the risk of CL+/- P but the precise mechanism is unknown. Intermittent hypoxia induced by nicotine may affect facial development (Bender, 2000, Castilla et al. 1999). A genetic predisposition (altered transforming growth factor [alpha]) may further escalate the risks of smoke exposure in select individuals (Shaw et al. 1996, Lorente et al. 2000).

- Maternal alcohol use, frequently associated with smoking, is also associated with an increased risk of CP (Lorente et al. 2000). Embryos exposed to alcohol had the migration and differentiation of neural crest cells interrupted (Bender, 2000). As with smoking, a genetic predisposition (alteration in the gene MSX1), coupled with exposure to more than 4 drinks daily, was associated with an increased risk of CL+/- P (Bender, 2000, Shaw et al. 1996, Lorente et al. 2000).

- Nutrition also plays an important role in the prevention of CL+/- P. Women of childbearing age should take 400 µg of folic acid per day, beginning before conception and continuing throughout pregnancy. Folic acid not only prevents neural tube and abdominal wall defects but also plays a role in the prevention of CL+/- P (Carinci et al. 2003, Murray, 2002, Cobourne, 2004).

Genetic influence on the other hand plays an important role in orofacial clefts and has been the subject of much research and discovery. Although the specific aetiology of CL+/- P and CP remains largely unknown, a strong genetic link is indicated (Stainer and Moore, 2004). Orofacial clefts are, therefore, characterised by familial aggregation, recurrence risks and elevated concordance rates in twins providing evidence for a genetic component in its aetiology (Mitchel and Risch, 1992). Although the risk of clefts is increased when a positive family history exists, no predictable pattern of recurrence has been established (Wantia and Rettinger, 2002). It has been established that orofacial clefts do not follow the rules of monogenic inheritance and that they demonstrate non-mendelian

transmission patterns. Reported rates of the recurrence risk are as follows (Tudose et al. 2007):

- Two healthy parents with an affected child have a general risk of 3.3% of getting another affected offspring.

- The recurrence risk triples (12 –14%) after the birth of two affected children.

- An affected parent has a risk of 4% to have an affected offspring. If a child with CL+/- P is borne, the risk for a new pregnancy will be 3-4 times greater then after two affected offspring the risk becomes 25%.

- A healthy person with an affected brother/sister has a risk of 3-7% of getting an affected offspring.

- The recurrence risks for isolated CP are slightly lower than those for CL+/- P.

- The risk seems to be influenced by the sex of the affected child being slightly increased for female affected offspring.

- The risk is also influenced by the severity of the clefts: the risk doubles for bilateral CL+/- P.

- General risk of recurrence for normal individuals in the population is 0.1% for CL+/- P and 0.04% for isolated CP.

According to Tolarova, (1990), from a clinical point of view, 2 factors are most important when evaluating the risk of recurrence for CL+/-P: the gender of the individuals (ie, patient and individual at risk) and the severity of the effect in the patient (eg, unilateral or bilateral). The lowest recurrence risk for CL+/-P is for the subcategory of male patients with unilateral cleft and, within this category, for sisters of males with a unilateral cleft and for daughters of fathers with a unilateral cleft lip with or without cleft palate. The highest risk of recurrence of CL/P is for the subcategory of female patients affected with a bilateral CL/P. The recurrence of CP seems only to be influenced by gender. The highest risk is for daughters of fathers affected with an isolated CP and the lowest for sons of mothers affected with an isolated CP.

The Tolarova (1990) study further explains that clefts are caused by an interaction between genetic and environmental factors, whereby the genetic factors create a susceptibility for clefts while the environmental factors trigger cleft development. The proportion of environmental and genetic factors varies with the gender of the individual affected with the cleft. In CL+/- P it also varies with severity of the cleft (bilateral or unilateral). The highest proportion of genetic factors was found in the subgroup of female patients with a bilateral cleft and the smallest proportion in the subgroup of males with a unilateral cleft. This study found that the subgroup of clefts closest to the population average (males, with unilateral clefts), had the highest population prevalence, the lowest value of heritability and the lowest risk of recurrence.

A lower risk of recurrence or heritability indicates a higher proportion of environmental factors. This gives a better chance to act in prevention because

the only aetiological factors that can be changed are the environmental factors. This has been confirmed by a large population based study by Tolarova (1990).

Several studies reveal that the following genes among others, may play a role in the aetiology of orofacial clefts: TBX22, PVRL1, MSX1, MTHFR, TGFA, TGFB3 and lately, Interferon Regulatory Factor 6 (IRF6) gene (Zucchero et al. 2004, Murray and Schutte, 2004, Maestri et al. 1997, Wyszinski et al. 1997, Scapoli et al. 1998, Blanco et al. 2001, Mitchell et al. 2001, Beaty et al. 2002, Kondo et al. 2002, Wong and Hagg, 2004). However, except for the IRF6 gene, confirmation of the contribution of the other implicated genes has been found to be inconsistent among various populations (Blanton et al. 2005).

The IRF gene encodes interferon regulatory factors that constitute a family of transcription factors that regulate the transcription of interferons. There are 9 reported IRFs in humans but the function of IRF6 is largely unknown though it may be involved in the regionalization of the mesoderm during craniofacial development (Yetter, 2002, Taniguchi et al. 2001). Mutation of the IRF6 gene causes the Van der Woude syndrome (VWS), an autosomal dominant disorder, which provides one of the best models for non-syndromic CL+/- P because it closely resembles it. The only additional feature in the syndrome is lower lip pits. Expression analyses carried out on animal models (mice) was reported to show high levels of the IRF6 mRNA along the medial edge of the fusing palate, tooth buds, hair follicles, genitalia and skin of embryonic and adult mice. These observations demonstrate that haploinsufficiency of IRF6 disrupts orofacial development and are consistent with dominant-negative mutations disturbing

development of the skin and genitalia. This is in accord with the VWS and Popliteal Pterygium Syndrome phenotypes. The Popliteal Pterygium Syndrome is a disorder with a similar orofacial phenotype to the VWS but also includes skin and genital anomalies (Kondo et al. 2002).

Since the phenotype of VWS directly overlaps with that of non-syndromic CL+/- P, the gene mutations causing the syndrome were isolated and found within chromosome 1q32-q41. It was then assumed that the mutations causing non-syndromic CL+/- P will be found within this chromosomal region that is the IRF6 gene. A direct sequence analysis of the IRF6 region was done and this approach was confounded by single nucleotide polymorphisms (SNPs). These are normal DNA sequence variants that occur at every 1900 base pairs. To distinguish between putative disease causing mutations and SNPs, which are normal, a pair of monozygotic twins who were discordant for the VWS phenotype and had unaffected parents were studied. The only sequence difference between the twins resulted from a somatic mutation in the affected twin (Kondo et al. 2002).

The IRF6 gene mutations have been reported to account for 12% of all non-syndromic CL+/- P compared to all the other genes which, together, account for 2% of the non-syndromic cases. One variation at the IRF6 gene has been reported to triple the risk of recurrence in families that had one affected child (Zucchero et al. 2004). Various studies in populations of Caucasian and Asian ancestry have been done to confirm the contribution of the IRF6 gene in the aetiology of non-syndromic cleft lip and palate. A study by Zucchero et al. (2004) detected an association between SNPs in IRF6 and non-syndromic CL+/- P in a

number of populations. Subsequent studies by Scapoli et al. (2005) confirmed this association in the Italian population while Blanton et al. (2005) confirmed that IRF6 plays a role in non-syndromic CL+/- P after studying a well characterised sample of non-syndromic CL+/- P families. No study has been done to determine the contribution of the IRF6 gene towards clefting within the Kenyan population.

It has been postulated that non-syndromic CL+/- P is phenotypically variable and genetically heterogeneous, as has been shown by the number of implicated genes (Blanton et al. 2005). It can, therefore, be deduced that gene-gene interactions occur and implicated genes may be involved in a common genetic pathway with each gene making a contribution to the overall risk. In support of a common pathway, Kondo et al. (2002) found two IRF binding sites in the promoter of MSX1 gene and one in the intron. Hence, it is important to note that although there is strong evidence supporting the involvement of the IRF6 gene in non-syndromic CL+/- P, it is unlikely that its polymorphisms alone are directly involved in the CL+/- P aetiology. Other genetic alterations increasing susceptibility to the disease are still unknown and more studies are needed in this area (Scapoli et al. 2005).

Gene-environment interactions have also been implicated in the aetiology of CLP. Studies of the role of maternal smoking with TGFA mutations and maternal alcohol use with MSX1 mutations as covariates suggested that these loci might be susceptible to detrimental effects of those environmental risk factors (Shaw et al. 1996, Beaty et al, 2002). Folate-metabolizing enzymes such as

methylenetetrahydrofolate reductase (MTHFR), which is a key player in the aetiology of neural tube defects and RFC1 are considered candidate genes based on data that suggest that folic acid supplementation can reduce the incidence of non-syndromic CL+/- P (Tolarova, 1995).

The purpose of this study was to determine the presence of an IRF6 gene variant in cases of non-syndromic CL+/- P and CP in indigenous Africans, considering the low incidence of the condition in this population. Both clefting phenotypes of non-syndromic CL+/- P and non-syndromic CP have been considered as separate entities in this study. The rs2013162 variant studied has been significantly associated with non-syndromic CL+/- P (Blanton et al. 2005, Zucchero et al. 2004, Scapoli et al. 2005). Assay information on the same variant from Applied Biosystems* also revealed that this variant had a minor allele frequency of 0.23 in African Americans. In view of this, it was postulated that this variant is most likely to be found in the indigenous African population. There is scarce information regarding the genes implicated in orofacial clefts in indigenous Africans and no studies to determine the contribution of the IRF6 gene to clefting in the Kenyan population have been done. The identification of factors that contribute to the aetiology of non-syndromic CL+/- P and CP is important for prevention, treatment planning, and education. With an increasing number of couples who seek genetic counselling as a part of their family planning, the knowledge of how specific genes contribute to the formation of non-syndromic orofacial clefts has gained increased importance.

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* Applied Biosystems, Foster City, CA, U.S.A.

1.1. STATEMENT OF RESEARCH PROBLEM AND JUSTIFICATION

Although orofacial clefts are not life threatening conditions, they can be remarkably disabling. Furthermore, their treatment is long-term in nature and often requires a multidisciplinary, team based approach. It is, therefore, expensive and out of reach for a majority of the patients in Kenya. When untreated, these conditions result in significant psychosocial trauma due to the facial deformities. Other complications caused by the conditions include feeding problems, speech and language delay, ear infections, hearing loss as well as dental problems.

The birth of a baby with an orofacial cleft is, therefore, an emotional event for parents. They may feel that they are to blame and often wonder how they can prevent it from occurring again. By understanding the emerging genetic links, environmental influences and potential teratogens that may interact to contribute to clefting, the healthcare professional can help parents understand how these defects occur and in many cases reassure parents that it is not their fault. As healthcare professionals provide the specific treatment, they need to assist in providing accurate information and family support along the way. This can be through educational resources and counselling.

There is a scarcity of information on the contribution of the IRF6 gene variations to clefting in the indigenous African population. Results obtained from this study will provide information that will advance the ongoing research on the contribution of the IRF6 gene to non-syndromic clefts. This study will help determine whether the IRF6 gene is a major genetic influence in our population, considering that the

indigenous Africans have the lowest prevalence of clefting. By so doing, the study will help advance the understanding of the aetiology of these conditions. Identification of the causative genes will also assist in determining any associated environmental factors and provide options for preventive measures.

1.2. OBJECTIVES

Broad objective

To determine the presence of an IRF6 gene variant (rs2013162) and prevalence of environmental risk factors among indigenous African Kenyan patients presenting with non-syndromic clefts of the lip and/or palate at selected hospitals.

Specific objectives

1. To determine the pattern of distribution of non-syndromic clefts of the lip and/or palate in a Kenyan population.
2. To determine the presence of the IRF6 gene variant (rs2013162) in patients with non-syndromic clefts of the lip and/or palate.
3. To determine the prevalence of other risk factors associated with non-syndromic clefts of the lip and or palate.

1.3. HYPOTHESIS

Null Hypothesis

There will be no association between the IRF6 gene variant and non-syndromic clefts in the indigenous African population.

1.4. VARIABLES

Variable	*Description*	*Scale of measure*
Socio-demographic variables		
Age	Age of parent in years Age of child in months	Continuous variable
Gender	Male or female	Binary variable
Birth Rank	Birth order of child in the family	Ordinal variable
Education level	Highest level attained: None, Primary, Secondary, College, University	Ordinal variable
Occupation	Type of work done: None, Self employed, Employed	Nominal variable
Independent variables		
IRF6 gene variant	rs2013162 (alleles 1, allele 2 or both)	Nominal variable
Positive family history of clefts.	Yes or No	Binary variable
Maternal smoking during pregnancy.	Yes or No	Binary variable
Maternal alcohol use during pregnancy.	Yes or No	Binary variable
Drug history during pregnancy	Yes or No	Binary variable
Dependent variables		
Orofacial clefts	Phenotype of cleft – type of cleft as seen clinically.	Nominal variable.

CHAPTER TWO

2.0. MATERIALS AND METHODS

Study Design

The study was a descriptive cross-sectional study carried out from January 2008 to January 2009.

Study Area

The study was conducted in Kenya which has a population of 36.1 million according to the Kenyan National Bureau of Statistics (2006 estimate). Administratively, Kenya is divided into 8 provinces. Using purposive sampling, 5 hospitals were selected where free treatment to patients with CL+/- P and CP was offered. This was in a collaborative effort between Operation Smile Mission with the Government of Kenya and Smile Train Mission with Gertrude's Garden Children's Hospital. The selected hospitals and provinces represented were:

- Meru District Hospital (Eastern Province)
- Nyeri Provincial General Hospital (Central Province)
- New Nyanza Provincial General Hospital (Nyanza Province)
- Kenyatta National Hospital (Nairobi Province)
- Gertrude's Garden Children's Hospital (Nairobi Province)

These hospitals are district and provincial hospitals which are referral centres for the tertiary hospitals within the provinces. Kenyatta National Hospital is the main referral hospital in Kenya and is situated in Nairobi, the capital city. Gertrude's Garden Children's hospital is a private children's hospital that offers specialist services.

Laboratory analysis was carried out at the Walter Reed Project-KEMRI laboratory, located in Nairobi, in close proximity to the Kenyatta National Hospital.

Study population

This comprised of unrelated patients with non-syndromic clefts with their accompanying biological parents. Where the father was not available, pairs of affected child with accompanying mother were recruited.

Criteria for patients

The inclusion criteria for the study were:

- Patients whose parents consented.
- Patients below the age of 18 years who gave assent.
- Those with non-syndromic CL+/- P or CP.
- Indigenous Kenyan Africans determined from ethnic tribes listed by the Kenya National Bureau of Statistics (Facts and Figures 2007).
- Those with biological parents or mother available for investigation.
- Those who did not have a relative already recruited in the study.

The exclusion criteria for the study were:

- Those who did not give consent or assent.
- Those with syndromic CL+/- P or CP.
- Those who were not indigenous Kenyan Africans.
- Those who were not accompanied by biological mothers.
- Those who had a relative already recruited in the study.

Sample design and procedure

The sample size was determined using the following formula (Daniel, 2006):

$$n = \frac{Z^2 P (1-P)}{d^2}$$

n=desired sample size when study population is >10,000

Z= standard error corresponding to 95% confidence level

d=degree of accuracy

P= prevalence of the IRF6 variations in non-syndromic CL/P – 12% as reported by Zucchero et al (2004).

$$\frac{1.96^2 \times 0.12(0.88)}{0.05^2} = 162$$

Therefore, n=162

Since the study population was below 10,000, the required sample size was determined using the following formula:

nf=desired sample size when study population is <10,000 = $\frac{n}{1+n/N}$

N=estimated population size (400 patients all 5 hospitals considered. A prevalence of 70% is expected to be non-syndromic therefore non-syndromic cases will be 280)

$$nf = \frac{n}{1+n/N} = \frac{162}{1+162/280} = 102$$

A minimum sample size of 102 child and parent triads or child mother pairs was required and a convenient sampling method was used to select all the patients and parents presenting at the study sites, within the study period, who met the inclusion criteria.

Data collecting instruments and technique

Data collecting instruments were designed in consideration of the recommendations of the 2003 WHO Registry Meeting report on Craniofacial Anomalies. A questionnaire and clinical examination chart were used to screen and select the non-syndromic patients who were included in the study (Appendix 3). This instrument ruled out syndromic cases based on:

1. History of syndromes in the family.
2. Use of drugs that are known teratogenic factors for syndromic cases (Phenytoin, Warfarin, Thalidomide and Retinoids)
3. Thorough clinical examination to rule out the presence of any somatic or neurological deficits by paediatricians, paediatric anaesthetists and paediatric intensivists. This clinical examination was also done to ensure that these children were fit for theatre.

One hundred and fifty one patients and their parent(s) were recruited for the study and put through the preceding screen test. Those who were found to have been syndromic after the clinical screen test were excluded from the study and thanked for participation in the study. One hundred and nineteen patients with their biological parent(s) remained in the study and were subjected to data collection as follows:

Data collecting instruments

For those who met the inclusion criteria the following data collection instruments were used:

1. Data collection sheet (Appendix 4) which entailed

A) A questionnaire that was administered by the investigator, to record:

- Demographic details of the child, mother and father.

  Those of the father were reported by the mothers interviewed.

- Family history of clefting, history of smoking or alcohol use in pregnancy and use of drugs in pregnancy.

B) An examination chart, to record:

- The phenotype of the non-syndromic clefts – A modification of the 1942 Fogh-Andersen classification was used (Fogh-Andersen, 1971). This is a morphological classification and represents the phenotypes that occur in clefting. It also separates the CL+/- P and CP which have been found to be morphologically and aetiologically different. It was modified for a better description of the type and laterality of the cleft and to help give an indication of severity. This classification was selected over the other classifications because of its simplicity and repeatability as well as its ability to capture the relevant information on phenotypes required in this study.

2. Sample collection swabs

These were part of the **BuccalAmp™ DNA Extraction Kits*** and were used to collect the buccal cell samples. In addition, the BuccalAmp DNA Extraction Kit also includes QuickExtract Solution for DNA extraction provided as individual sample tubes. The BuccalAmp DNA Extraction Kits were transported and delivered from the manufacterer in dry ice. Upon arrival, the

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* EPICENTRE Biotechnologies, Madison, Wisconsin, U.S.A.

tubes of QuickExtract™ DNA Extraction Solution were stored in the Walter Reed Project (KEMRI) laboratory at 4°C and the Sterile Sample Collection Swabs at room temperature.

3. **A laboratory form which was used to record**

- The presence of single marker mutations (alleles) in patient and parent(s).

Data Collecting Technique

A) Sample collection

For collection of the samples, only the swabs were carried to the field. The collection swabs provided gentle, safe buccal sample collection, even for infants. The swabs were provided individually packaged in sterile hard-pack plastic cylinders. Sample collection was done by rotating the swab on the inside of the cheek approximately 20 times as recommended in the product protocol. After collecting the sample, the sample swab was air dried for 10-15 minutes at room temperature. The dry swab was then returned to the cylinder package, for safe, secure storage between 22-37°C and transported from the collection site to the Walter Reed-KEMRI Project laboratory within the week. Meticulous labeling of the samples was ensured (samples are stable for up to 1 week at temperatures of up to 37°C). Any samples that did not have clear labeling were discarded. Six samples were discarded, leaving 113 samples.

For the laboratory procedure to extract DNA and to amplify and detect the mutation of interest (rs2013162), a few samples were first prepared and run as a trial run under supervision to ensure that quality assurance was observed at all

times. After the trial run, the supervision was continued until all the samples had been run.

In order to obtain PCR-ready DNA at the laboratory, the QuickExtract solution tubes were labeled with the corresponding number on the swab. Buccal sample swab was placed in the tube containing the extraction solution and rotated a minimum of 5 times. The cap on the tube was then screwed tightly and the mix vortexed for 10 seconds. Incubation of the tube was then done at 65 ^{o} C for 1 minute, the mixture was vortexed for 15 seconds then the tube was incubated at 98^{o} C for two minutes. The mixture was then finally vortexed for 15 seconds before storage at -70 ^{o} C. Sample collection and DNA extraction was done in accordance with the Epicentre Biotechnologies protocol.

B) PCR Amplification

The IRF6 gene variant of interest in this study was rs2013162. This variant is known to cause a silent mutation. These are mutations that do not result in a change of the amino acid sequence of a protein. The protein product is, therefore, not changed but it has been shown that silent mutations can affect the folding of a protein or cause alternate splicing thus changing the function of the protein formed. The SNP alleles for this variant are [A/C] with a forward design strand (the probe binds to the forward strand). Therefore, the SNP alleles on the sample when it is positive for both alleles are [T/G]. The amplification and determination of the presence or absence of rs2013162 was done following the Applied Biosystems protocol.*

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* Applied Biosystems, Foster City, CA, U.S.A.

Reagents and Storage

SNP Genotyping Assays* and TaqMan Genotyping Master Mix* were obtained. These were transported and delivered in dry ice to prevent freeze thaw cycles. The assays were packed in opaque packets to prevent exposure to light as this affects the fluorescent probes. The SNP Genotyping Assays were stored at -15°C to -25°C in the dark. The TaqMan Genotyping Master Mix was stored at 2°C to 8°C.

Reagents and Sample Preparation

- The SNP Genotyping assays were diluted from a 40× concentration to a 20× working stock with 1× TE buffer.* This diluent was then vortexed and centrifuged. Multiple aliquots of the same were then made and stored at -15 to -25°C. This allowed only the assays in use at any one time to be thawed, preventing freeze thaw cycles.
- SNP Genotyping Assays in use were resuspended by vortexing. Following this, the tubes were centrifuged briefly.
- The TaqMan Genotyping Master Mix was thoroughly mixed by swirling the bottle.
- Frozen DNA samples were thawed in ice and resuspended by vortexing then centrifuging the tubes briefly.

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* Applied Biosystems, Foster City, CA, U.S.A.
Constituents of the SNP Genotyping Assay are indexed (Appendix 5).

* 1× TE buffer constituents: 10Mm Tris- HCl, 1Mm EDTA at a pH of 8.0 and made with DNase-free, sterile filtered water.

Preparation of the Reaction Mix

The reaction mix is made from 20× SNP Genotyping Assay, TaqMan Genotyping Master Mix and DNase-free water if dry DNA samples are used. When wet DNA samples are used, no DNase-free water is added. This study used wet DNA samples, therefore, no DNase-free water was added when making the reaction mix.

The recommended volume per well for wet DNA is 13.75μL in a 96 well reaction plate, and this was used in this study. To prepare the reaction mix, the following steps were followed:

- The number of reactions to be performed was calculated for each assay. Two No Template Controls (NTCs) were included per assay. For these DNase-free water is used in place of the DNA sample. This orients the VIC-dye and FAM-dye clusters to an origin and enables detection of DNA contamination on a given set of plates.
- The total volume of each component needed was calculated using the volumes required per well (Table 1). Some extra reactions were included in the calculations to compensate for volume loss during pipetting.
- The tube was then centrifuged to spin down the contents and to eliminate any air bubbles from the reaction mix.

Table 1: Constitution of the Reaction Mix.

Components	Wet DNA Method 96-well plate (μL/well)
TaqMan Genotyping Master Mix	**12.50**
20× SNP Genotyping Assay	**1.25**
DNase-free water	**None**
Total volume per well μL/well	**13.75**

- The reaction mix was then pipetted into the wells of a MicroAmp™ Fast Optical 96-well Thermal Cycling Plate* with the volumes indicated per well as shown in Table 1. For this study, 13.75μL was dispensed per well.
- All the wells were inspected for uniformity.
- A wet DNA sample was then pipetted into each well. The volume pipetted was 11.25μL as recommended in the protocol. Care was taken to prevent cross contamination during pipetting.
- The plates were then sealed with a MicroAmp™ Optical Adhesive Film.*
- The plates were vortexed to mix the contents in the wells and centrifuged briefly to spin down the contents and eliminate air bubbles.

Performing PCR

To perform PCR, a 7500 Fast Real Time PCR system* was used. The following steps were followed:

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* Applied Biosystems, Foster City, CA, U.S.A

- The thermal cycling conditions were specified as shown in Table 2.

Table 2. Thermal cycling conditions for Realtime PCR.

Amplitaq Gold Enzyme Activation	PCR (50 cycles)	
HOLD	Denature	Anneal
10 min at 95^0C	15 sec at 92^0C	90 sec at 60^0C

- The reaction volume (25μl/well) was specified.
- The reaction plate was loaded onto the Realtime PCR machine and the run started.

Results

The results were read manually. Positive samples were taken as any sample that amplified within a 15-35 threshold cycle as these could be sequenced (Dorak, 2006).

Validity and reliability

The principal investigator was calibrated by one of the supervisors (Dr Tom, M. Osundwa) to measure the inter-examiner reliability on the classification of non-syndromic cases. Cohen's Kappa value was used to calculate reliability. A Kappa value of 1 for inter-examiner reliability was obtained which showed consistency and no variations in classification of clefts. A repeated examination in assessing intra-examiner reliability in classification of non-syndromic clefts was not carried out as the Cohen's Kappa score for inter-examiner reliability showed consistency

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with no variations. Also, logistics at the sites could not allow for this repeated examination by the principal investigator. In the laboratory, after amplification of all the samples, a final reaction plate was prepared to repeat amplification of samples randomly selected from previous plates. This was to test for reliability of the PCR process. A Kappa value of 0.78 which is acceptable was obtained.[62] For further reliability, standardized primers and probes and genotyping assays were obtained from Applied Biosystems (Blanton et al. 2005, Zucchero et al. 2004, Scapoli et al. 2005).

To ensure that the findings were valid, a calibrated 7500 Fast Realtime PCR machine* was used to amplify the samples. This PCR machine was last calibrated in November, 2008 and is due for calibration in May, 2009. Protocols of sample collection and DNA extraction were adhered to. For the PCR process ABI protocol was strictly adhered to.

Data analysis and presentation

Data were coded, entered into a computer and analyzed using SPSS version 12.0 and Epi-Info version 3.32. Measures of centrality and variance were computed for the continuous variable. Frequency distribution and proportions were used for the description of findings. The Chi square with Yates correction test and Wilcoxon Signed Rank's test were used to determine the relationship between the variables. Data were presented using bar and linear graphs and a pie chart.

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* Applied Biosystems, Foster City, CA, U.S.A.

Minimising biases

To minimise selection bias,

- Only those participants who met the inclusion criteria were enrolled for the study.

To minimise measurement bias,

- The questionnaire was pre-tested.
- The investigator was calibrated.
- A clearly written protocol was used to standardize the procedure for buccal cell sample collection. This included collection of the sample, transport and storage.
- Meticulous labelling of samples was done.
- Standardized methods and techniques (protocols) were used for the laboratory process.
- Repeated testing at the laboratory was done.
- Procedure for determining the IRF6 gene variation was validated.

Ethical considerations

The proposal was submitted to the Kenyatta National Hospital and University of Nairobi Ethics, Research and Standards Committee (Approval Number P1/1/2008) and the Ethics, Education and Research Committee at the Gertrude's Garden Children's Hospital for approval (Appendix 6). Permission was sought from the Operation Smile Mission in Kenya, Smile Train Mission and all the hospitals involved (Kenyatta National Hospital, Meru District Hospital, Nyeri Provincial General Hospital, New Nyanza Provincial General Hospital and Gertrude's Garden Children's Hospital). The purpose of the study, the expected

benefits and risks were explained to the parents. All details were contained in the consent form. A written informed consent was then sought and obtained from the parents to join the study and to publish the data arising from their participation. The sample collection technique was atraumatic and was carried out under strict sterile conditions. DNA was extracted from buccal cells that were obtained using sterile swabs. The data obtained were confidential with no names included and participation was voluntary, with participants at liberty to terminate participation without victimization. Paternity was not checked as only 3 children were accompanied by both parents while a majority were accompanied by their mothers only. The samples from the fathers though examined, were not considered significant. In the event that paternity tests were to be carried out, this information was confidential as no names were included in the study. Results obtained were for the benefit of those affected by CL/P.

Limitations

- The study time frame could not allow for a large population based sample to be investigated.
- A non-probability sampling method was used for convenience and, therefore, the sample was biased.
- The reagents used were imported from overseas and required strict temperature controls and adherence to time frames during delivery and clearance. In the present study, reagents were ordered several times when these temperature or time limits were breached. Adequate time and money should, therefore, be set aside for this likely eventuality.

CHAPTER THREE

3.0. RESULTS

3.1. SOCIO-DEMOGRAPHIC CHARACTERISTICS

One hundred and fifty one patients were screened during the study. Of these, 32 were found to have been syndromic and, therefore, excluded from the study. During laboratory analysis, another 6 samples were found to have had unclear labelling and were discarded, leaving 113 patient and parent samples. This study therefore, included 113 children with orofacial clefts among whom 65 (57.5%) were males and 48 (42.5%) females. The age of presentation ranged between 1 month and 15 years with a mean of 26.46 months (± 37.41 S.D). The majority of these children (99, 87.6%) were in the 0-5-year age group. According to the age and gender pattern of the study participants there were more males than females in the 0-5-year age group while in the 6-10- and 11-15-year age groups there was a female preponderance (Fig. 1). There was no statistically significant difference in the distribution of the children with clefts according to age between the males and the females (χ^2 Yates correction= 2.17, p=0.1402).

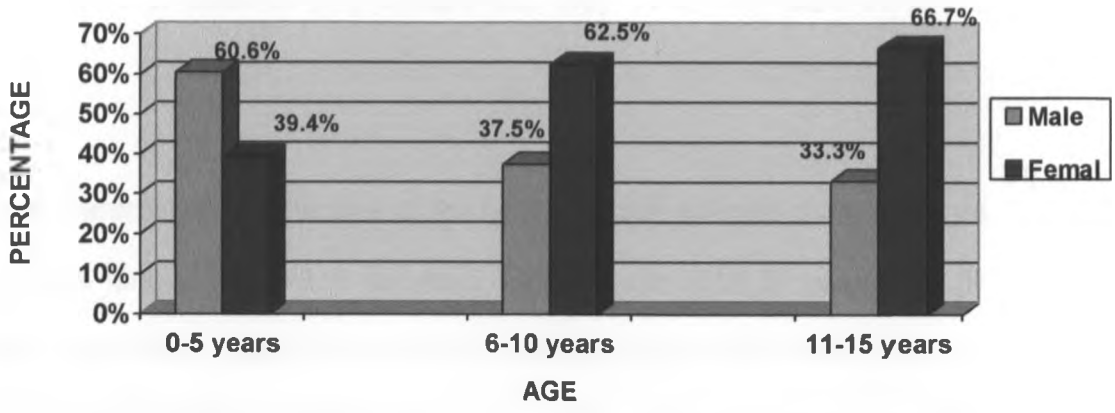


Fig. 1: Distribution of the children with clefts according to age group and gender.

Remarkably, majority of the children (41, 36.3%) were in the 1st birth rank. Fig. 2 summarizes the distribution of the children in the study according to birth rank.

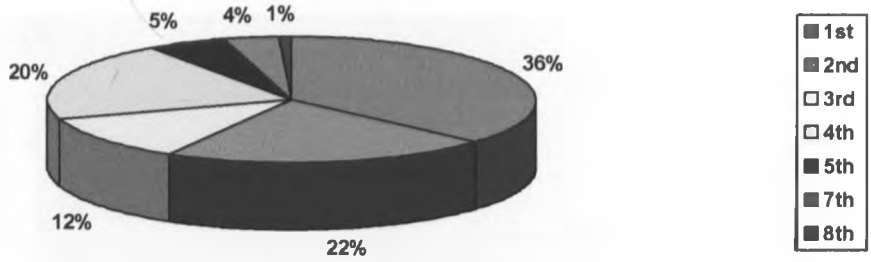


Fig. 2: Distribution of the children according to birth rank.

The ages of the mothers ranged from 18 to 45 years with a mean of 28.7 years (± 5.96 S.D) while those of the fathers ranged from 21 to 60 years with a mean of 34.09 years (± 7.97 S.D). Majority of the mothers in the study (105, 93.8%) had received some formal education and; most (62, 54.9%) were unemployed while

40 (35.4%) were in informal employment and only 11 (9.7%) were in formal employment.

Considering parental age at the time of the birth of a cleft afflicted child, the ages of the mothers ranged from 15 to 45 years, with a mean of 26.57 years (± 5.57 S.D) while those of the fathers ranged from 17 to 52 years with a mean of 31.96 years (± 7.54 S.D). Majority of the mothers (78, 69%) were below the age of 30 years during the birth of a cleft afflicted child while majority of the fathers (62, 54.9%) were above the age of 30 years. Fig. 3 summarizes the distribution of parents within the less than 30-year and more than 30-year age groups. Evidently, women were significantly younger than men at the birth of a cleft afflicted child (Z= - 8.766; p=0.000).

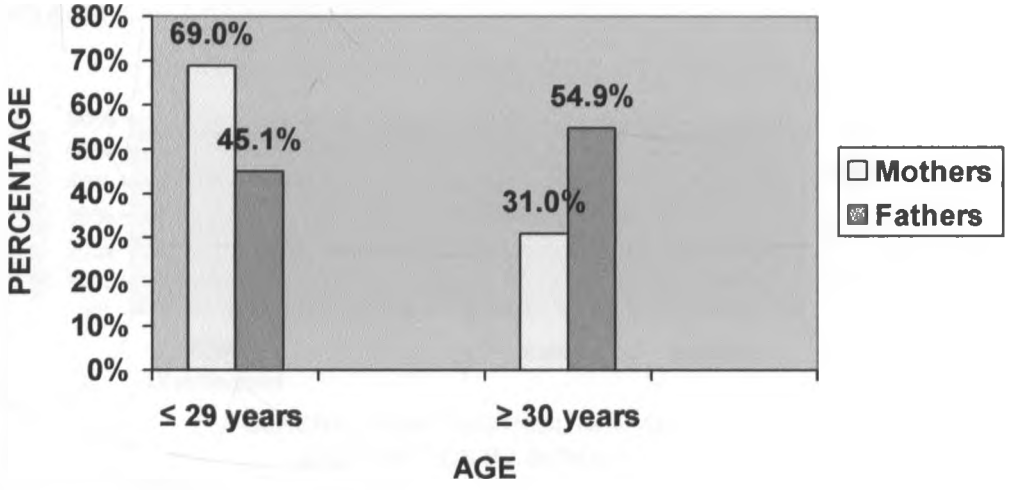


Fig. 3: Distribution of parents according to age.

With regard to parity, most of the mothers (72, 63.7%) were multiparous while 41 (36.3%) were first time mothers. Of the first time mothers, majority (39, 95.1%)

were less than 30 years of age while only 2 (4.9%) were 30 years and above (elderly primigravida) at the birth of the cleft afflicted child. The first time mothers were significantly younger than the multiparous women (χ^2 with Yates correction=18.62, p=0.0000).

3.2. PATTERN OF DISTRIBUTION OF CLEFTS

On the distribution of the various types of clefts, 61 (54%) of the children had CL, 48 (42.5%) had CLP while only 4 (3.5%) had isolated CP. Fig. 4 shows the distribution of the types of cleft according to gender. Isolated CL was the most common phenotype in both males and females. A female predilection for the CL and CP phenotypes was noted while a male predilection was observed for the CLP phenotype. No statistically significant difference in the distribution of cleft types between the males and females was noted (χ^2 Yates correction=0.21, p=0.648).

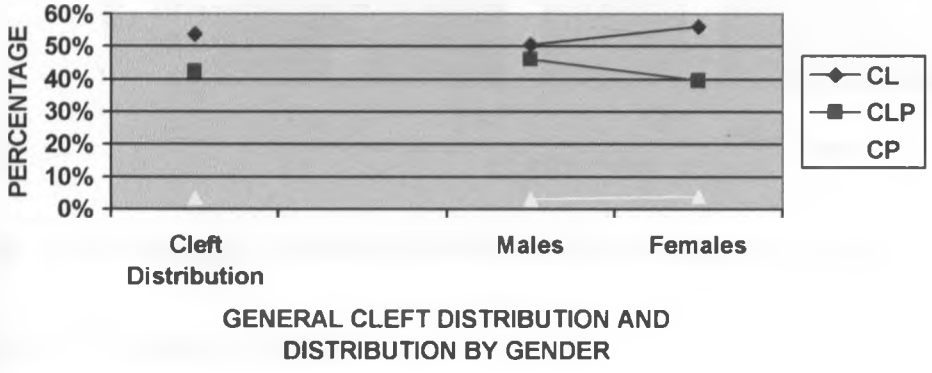


Fig. 4: Distribution of cleft types according to gender.

On laterality of the clefts, majority (79, 70%) were unilateral while 34 (30%) were bilateral. Considering the specific cleft types, for the CL phenotype, 54 (88.5%)

were unilateral while 7 (11.5%) were bilateral clefts. Of the unilateral clefts, most (36, 66.7%) were left sided. On the other hand, the CLP phenotype was equally distributed between the unilateral (24, 50%) and bilateral clefts (24, 50%). Of the unilateral clefts, majority (15, 62.5%) were also left-sided. The CP phenotype was diagnosed in only four children. One was a unilateral right sided CP while the other 3 were bilateral clefts. Fig. 5 summarizes the status of cleft laterality in this population. Considering laterality and gender, the most common phenotype observed was the unilateral left-sided CL with a male predominance. Indeed, a male predilection was diagnosed for all cleft types within the CL+/- P phenotypes except the unilateral right-sided CL.

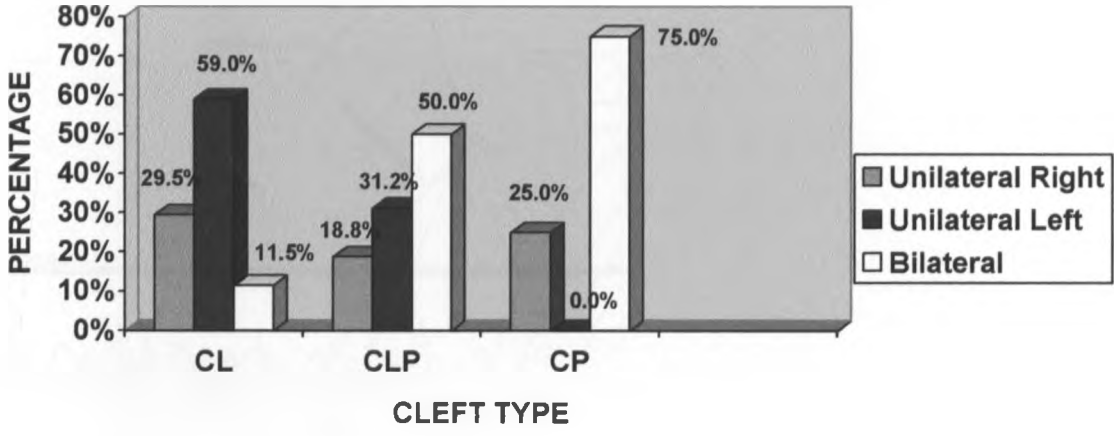
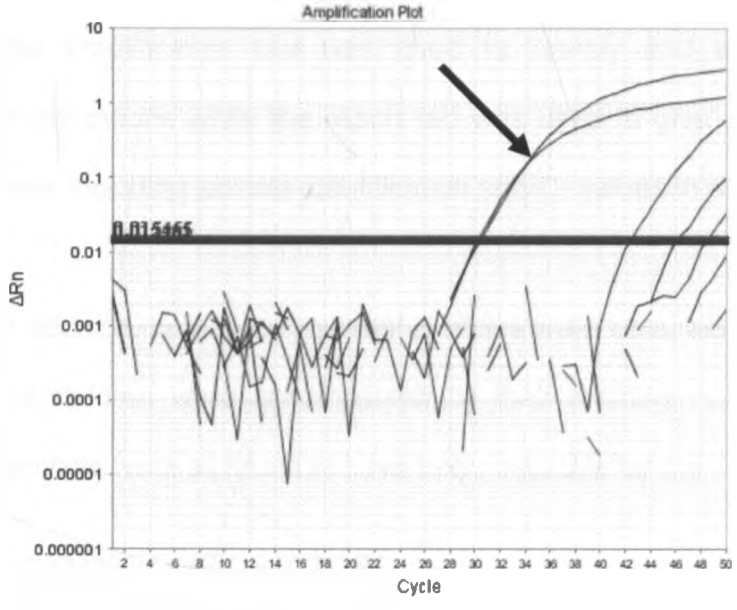


Fig. 5: Distribution of clefts according to phenotype and laterality.

3.3. IRF6 GENE VARIATION

The presence of the IRF6 gene variant (rs2013162) was determined in only 3 (2.7%) of the children included in this study. One male child was heterozygous for the G and T alleles and had unilateral CLP while two children, one male and one female were both homozygous for the G allele and had unilateral CL. These children had no family history of clefting and had not been exposed to any of the

other risk factors studied. Three mothers in the study (2.7%) were also found to have been positive for the IRF6 gene variant. Of these mothers, only one was a parent of a child positive for the same variant. This mother-child pair was homozygous for the G allele. The other mothers with the gene variant were both homozygous for the T allele. The transmission disequilibrium test (TDT) could not be done to test for an association between the IRF6 gene variant and non-syndromic clefts as the positive samples obtained were too few. Fig. 6 and Table 3 show a sample of the laboratory results generated by the PCR process. A positive sample was taken as that with amplification within a 15-35 threshold cycle. The positive sample illustrated by the arrow in Fig. 6 and highlighted in Table 3 is positive for both allele 1 and 2 (heterozygous for the G and T allele).



ΔRn – Delta Normalized Reporter.

Fig. 6: Amplification plot of wells G1-G10 (PCR run 10).

Table 3. Report Tab of wells G1-G10 (PCR run 10).

Well	Sample	Targets	C_T value
G1	90M	1	46.123
G2	90M	2	42.553
G3	90C	1	30.198
G4	90C	2	30.570
G5	92M	1	Undetermined
G6	92M	2	48.129
G7	92C	1	Undetermined
G8	92C	2	Undetermined
G9	93M	1	Undetermined
G10	93M	2	Undetermined

Undetermined: No amplification by the cut off point (50th cycle).
Target 1 (VIC dye) positive: Homozygosity for allele 1 (G allele).
Target 2 (FAM dye) positive: Homozygosity for allele 2 (T allele).
Target 1 and 2 positive: Heterozygosity for allele 1 and 2 (G and T allele).
C_T: Threshold Cycle.

The amplification plot was used to identify and examine for any irregular amplifications while the report tab was used to give detailed information of the wells including sample identification and C_T values in a table form.

In addition, only three paternal samples were obtained in the study as majority of the children were accompanied by their mothers only (110, 97.3%). These 3 samples were all found to have been negative for the IRF6 gene variant tested.

3.4. OTHER RISK FACTORS

Only one mother (0.9%) gave a history of cigarette smoking in pregnancy and 4 (3.5%) gave a history of alcohol use during pregnancy. The phenotype of the only case where there was a history of maternal smoking was a unilateral CL. The 4 cases with a positive history of maternal alcohol use were distributed as follows: 3 cases of unilateral CL and 1 case of unilateral CLP.

On prescription drug history during pregnancy, 28 (24.8%) mothers reported to have taken drugs during pregnancy. Remarkably, drugs that were most commonly taken during pregnancy were antimalarials (23, 82.1%) and antibiotics (5, 17.9%). All the mothers who reported to have taken antibiotics took them during the first trimester while those who took antimalarials took them during all the three trimesters but mostly within the first and second gestational periods. Fig. 7 summarizes the distribution of those with a positive drug history according to type of cleft, laterality of clefts and gender. Among those with a positive drug history, the most common phenotype was CL (15, 53.6%). Those with CLP were 13 (46.4%) while none had CP phenotype. Considering laterality and gender, unilateral clefts were the majority (19, 67.9%) while 9 (32.1%) had bilateral clefts and the male gender was predominant (15, 53.6%) while the female patients were 13 (46.4%). However, there was no statistically significant difference in the distribution of clefts by phenotype (χ^2 Yates correction=0, p=0.9693) and gender (χ^2 Yates correction=0.07, p=0.789) but there was a statistically significant difference in the distribution of clefts by laterality (χ^2 Yates correction=7.26, p=0.007) between the participants with a positive drug history in pregnancy and those without.

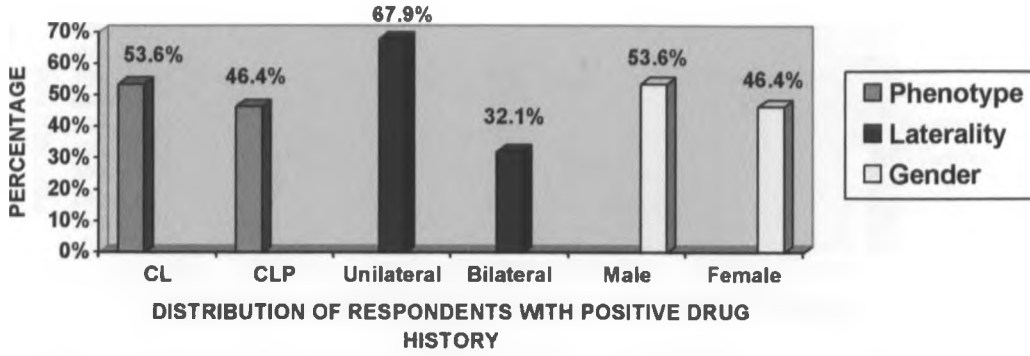


Fig. 7: Distribution of clefts according to phenotype, laterality and gender among the participants with a positive drug history.

A positive family history of clefting was reported in 16 families (14%) and all the affected family members were from the extended family. With respect to phenotype, cases that had positive family history of clefting were mainly of the CL phenotype (8, 53.6%). Considering laterality and gender, unilateral clefts were the most common (11, 67.9%) and a male predominance was noted (8, 53.6%). Fig. 8 shows a summary of the distribution of respondents with a positive family history by phenotype, laterality and gender. There was no statistically significant difference in the distribution of clefts by phenotype (χ^2 Yates correction=1.57, p=0.209), laterality (χ^2 Yates correction=1.04, p=0.3068) and gender (χ^2 Yates correction=0.03, p=0.871) between those with a positive family history and those without.

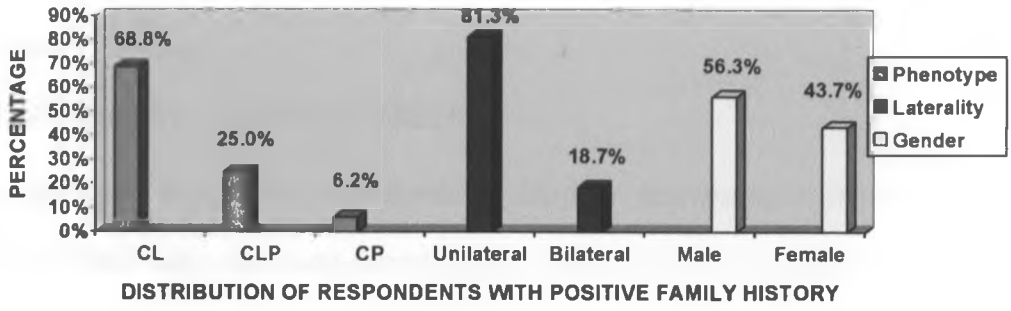


Fig. 8: Distribution of clefts according to phenotype, laterality and gender among the participants with a positive family history.

CHAPTER FOUR

4.0. DISCUSSION

4.1. SOCIO-DEMOGRAPHIC CHARACTERISTICS

Although not statistically significant, but similar to studies elsewhere, a male predominance in children with clefts was reported (Osundwa, 2005, Onyango and Noah, 2005, Wachira, 2009, Orkar et al. 2002). In Kenya, this may be a reflection of the normal population distribution according to age and gender. The Kenyan National Bureau of Statistics (Facts and Figures 2007) reports that there are more males than females in the 0-4-year age group in which majority of the children in the present study fall. The mean age of presentation in the present study was 26.46 (± 37.41 S.D) months. Onyango et al. (2005) and Donkor et al. (2007) both reported an early age of presentation, averaging 10 months while Osundwa et al. (2005) reported a mean age of presentation of 58.08 months (± 79.92 S.D) (Osundwa, 2005, Onyango and Noah, 2005, Donkor et al. 2007). The discrepancy in the ages of presentation observed could be attributed to the study sites. The latter study was carried out mainly in the rural areas of Kenya in missions offering free treatment while the former studies were carried out in an urban hospital setting with specialist services. The level of awareness of the condition, availability of treatment facilities and accessibility to treatment was probably better in the urban area hence the earlier and late ages of presentation in the preceding respective studies.

The present study was carried out in both rural and urban sites which may explain a mean age of presentation between that of the earlier studies. Similar to Onyango and Noah, (2005) the present study found majority of the children within

the 0-5-year age group, confirming that children with orofacial clefts indeed presented early.

With regard to birth rank, majority of the children in the present study were of the first birth order which was similar to the findings of Onyango and Noah, (2005) where a relatively young maternal age was inferred as parental ages had not been recorded. The present study indeed reported an early maternal age. Generally, 69% of the mothers were below the age of 30 years at the time of the afflicted child's birth. This was consistent with the findings of Donkor et al. (2007) who reported that 70% of the mothers in a Ghanaian study were below the age of 30 years. Maternal age in the present study was also similar to that in the general Kenyan population as reported by the Kenya Demographics and Health Survey of 2003. In addition, only 2 of the first time mothers in the present study had an advanced maternal age of 30 years and above at the time of a cleft afflicted child's birth. These findings may discount advanced maternal age and first pregnancies at an advanced maternal age (elderly primigravida) as risk factors to congenital malformations and; in particular clefting (Thigpen and Kenner, 2003, Lee, 1970). Notably, only 2 of the mothers were aged less than 19 years which may also discount maternal age of less than 19 years as a risk factor for clefting as reported by DeRoo et al. (2003). However, it is important to note that the sample in the present study is not representative of the entire population and therefore these findings should be subjected to further analytical studies with broader population samples.

With regard to paternal age, fathers were found to have been significantly older than the mothers. The Kenyan Demographics and Health Survey of 2003 reports a younger paternal age range of 15-54 years in the general Kenyan population compared to a paternal age range of 21-60 years in the present study. This may imply that increasing paternal age may be a risk factor to clefting. However, since most fathers were absent during data collection, this paternal age was reported by the mothers and may not be accurate. The sample size in the present study is also not representative of the entire population. This finding should, therefore, be subjected to analytical studies with larger population samples that include fathers during data collection. Similarly, Hodgkinson et al. (2005) and Thigpen and Kenner (2003), have reported increasing paternal age as a risk factor to clefting and more so if the maternal age is also more than 30 years of age.

With regard to education level, majority of the mothers in the present study had received formal education which is similar to the findings of the Kenyan Demographics and Health Survey 2003. This survey reports that as the mother's educational level rises, so does the likelihood that she would see a health professional for care during pregnancy. Therefore, this group of women would probably benefit from preventive measures administered through the antenatal care clinics. Most of the mothers in the present study were unemployed or in informal employment which was consistent with the findings in a Ghanaian study where most of the women were in low income occupations (Donkor et al. 2007). This may have had an impact on the care of these mothers during pregnancy. They would have been less likely to have had access to the perinatal use of

multivitamins and folate supplements which offer protection against clefting and other birth defects leaving them susceptible to the same (Tolarova, 1990).

4.2 PATTERN OF DISTRIBUTION OF CLEFTS

The pattern of distribution of clefts in the present study was similar to that of other African studies where CL is the predominant cleft type, followed closely by CLP with a few cases of isolated CP (Iregbulem, 1982, Orkar et al. 2002, Adeola et al. 2003, Donkor et al. 2007, Pham and Toleffson, 2007). Other Kenyan studies reported the CLP phenotype as the most common phenotype (Osundwa, 2005, Onyango and Noah, 2005). Wachira et al. (2009) in a Kenyan study with a retrospective and prospective arm reported CLP as the most prevalent type in the retrospective arm and CL as the most prevalent type in the prospective arm. These differences may be due to a change in the trend in the Kenyan pattern of distribution of clefts. Alternatively these differences could be attributed to different methodologies that may have been employed. The current study was highly selective, studying the non-syndromic cases only while the other studies included the syndromic cases. It should also be noted that the difference in frequency of the CL and CLP phenotypes is usually small and may not be significant. Larger population studies are, therefore, advocated for to harmonise the pattern of distribution of clefts in the Kenyan data. Usually, the African data show very low proportions of the CP variant (Iregbulem, 1982, Orkar et al. 2002, Adeola et al. 2003, Osundwa, 2005, Onyango and Noah, 2005, Donkor et al. 2007, Wachira, 2009). The Arab and Asian populations show higher proportions of the CP variant while in Caucasians, the pattern of distribution is consistent with CLP accounting for 50% of the cases, CL accounting for 25% and CP occurring in 25% of the

cases (Aljohar et al. 2008, Tan et al. 2008, Regezzi et al. 2008). These variations in the pattern of distribution of clefts may be racial or due to different environmental exposures based on geographical distribution.

Regarding the pattern of distribution of clefts with respect to gender, African studies show a varied pattern (Table 4). This difference in the distribution of clefts according to gender may be as a result of different methodologies used. It may also be attributed to regional distribution with exposure to different environmental influences. Asian and Caucasian data, on the other hand, report consistent findings with a male predominance for the CL and CLP cases and a female predominance for the CP cases (Tan et al. 2008, Tolarova, 1990). The reason for this pattern of distribution based on gender is not known. However, it has been postulated that gender differences in the incidence of CP may be related to differences in the timing of palate development. The palatal shelves move horizontally and begin to fuse by the seventh week in a male foetus. This does not occur until eight and a half weeks in a female foetus, providing a longer window of vulnerability to teratogens (Yetter, 2002).

Table 4. Distribution of cleft types according to gender in African studies.

Study	Study design	Study area	Distribution of clefts according to gender
Osundwa, 2005	Prospective, hospital based descriptive study	Rural areas, Kenya	Male predilection for all cleft types (n=396).
Onyango and Noah, 2005	Retrospective, hospital based descriptive study	Nairobi, Kenya.	Male predilection all cleft types (n=309).
Beston and Fabian, 2007	Retrospective, hospital based descriptive study	Dar es salaam, Tanzania	Male predilection for all cleft types (n=13).
Donkor et al. 2007	Prospective, hospital based descriptive study	Kumasi, Ghana	Male predominance for CL, equal distribution for CP, female predominance for CLP (n=74).
Wachira, 2009	Retrospective and prospective hospital based descriptive study	Nairobi, Kenya	Retrospective arm: Male predilection for CLP and CL, female predilection for CP (n=660). Prospective arm: Male predilection for CLP and CL, equal distribution for CP (n=68).
Present study	Prospective, hospital based descriptive study	Urban and Rural areas, Kenya	Male predilection for CLP, female predilection for CL and CP (n=113).

n= sample size

With respect to laterality, unilateral clefts were evidently, the most common, similar to most reports. Another consistent finding was that left-sided clefts were the majority (Osundwa, 2005, Orkar et al. 2002, Tolarova, 1990, Donkor et al. 2007, Iregbulem, 1982). Laterality of clefts has been a subject of much research but the findings have been inconclusive. Farina et al. (2002) reported that right- and left-sided clefts do not occur together in the same family and, therefore, may be under genetic control. They specifically reported a statistically significant distribution of linkage to chromosome 6 when compared with the homologous left

side. On the other hand, Masuzaki et al. (2004) in a study of monozygotic conjoint twins discordant for laterality of the cleft lip suggested that cleft laterality in this case was more affected by the process of twinning than by genetics since both twins had a normal 46,XY karyotype. It has also been postulated that different genes have different expressivity on the right and left sides during embryonic development and this may be a basis for the laterality patterns seen in clefting where multiple genes may be involved in the aetiology (Levin, 1997). Other studies have suggested that cleft laterality may be related to handedness and its basis may be embryonic in nature while others have discounted this claim by finding no statistically significant relationship between side of CL and handed dominance (Yorita et al. 1988, Jeffery and Boorman, 2000).

4.3. IRF6 GENE VARIATION

The presence of the IRF6 gene variant studied (rs2013162) was reported in only 3(2.7%) of the children included in this study. Three mothers were also found to have been positive for this gene variant and only one of them was a parent of a child positive for the same variant. Only 3 fathers were present during data collection and these, though too few to make any inferences, were negative for the IRF6 gene variant. Scapoli et al. (2005), Blanton et al. (2005) and Ghassibe et al. (2005) studied the same variant and reported that significant linkage disequilibrium was apparent for this marker in their populations. Transmission disequilibrium studies could not be carried out in the present study as the positive samples obtained were too few. The presence of this gene variant in the black African population studied is an indication that there is need for a larger black

population study which can test the association of this variant with non-syndromic clefts. However, the small numbers with this variant may suggest that perhaps this gene variant is not a major contributory factor to non-syndromic clefts in the population studied. The children with the IRF6 gene variant had unilateral forms of CL+/- P and two were male. Though the number of children was too small to make any inferences on the distribution of that gene variation by phenotype or gender, those affected had less severe forms of clefting (unilateral as opposed to bilateral clefts) and two were male.

Tolarova et al. (1990) in a large population based study showed that the proportion of environmental and genetic factors in the aetiology of clefts varies with the sex of the individual with the cleft and also varies with the severity of the cleft. From their large population study, it was emergent that the highest proportion of genetic factors was found in the subgroup of females with bilateral clefts and the smallest in the subgroup of males with unilateral clefts and vice versa for the environmental factors. Based on the Tolarova et al. (1990) study, findings in the present study may indicate that even though a genetic influence exists, there could be a higher influence from environmental factors. A higher proportion of environmental factors indicates a lower risk of recurrence and gives a better chance to act in prevention. It is, therefore, possible that the contribution of this gene to non-syndromic clefts may not be causal. Variations of the gene may contribute to clefting by increasing the susceptibility of those who have it to environmental factors. This warrants further investigations. Notably, the children with the gene variant had no history of clefting in the family. This is not surprising because studies done on familial non-syndromic orofacial clefts reported no

evidence of association of IRF6 gene variations with familial clefts (Birnbaum et al. 2008, Pegelow et al. 2008). This may be an indication that the IRF6 gene variations may be somatic mutations and are, therefore, not responsible for familial clefts. In the present study it was also noted that those positive for the IRF6 gene variation had not been exposed to any of the other risk factors associated to clefting in the study. It is possible that the IRF6 gene mutation is not a covariate with any of the known environmental risk factors. No known environmental factor has been associated to the IRF6 gene variations. The function of the IRF protein and mechanism of action of these variations in clefting is unknown which warrants further investigation (Blanton et al. 2005, Scapoli et al. 2005).

4.4 OTHER RISK FACTORS

Only one mother gave a history of cigarette use and 4 gave a history of taking alcohol during pregnancy. Donkor et al. (2007) reported no history of cigarette smoking or alcohol use in the Ghanaian population studied. This low prevalence may be due to under-reporting as mothers may not have wanted to admit to something that they felt might have caused the clefting. In contrast, mothers of children with clefts in Thailand and Malaysia admitted to smoking during pregnancy (Chuangsuwanich et al. 1998, Boo and Arshad, 1990). In the present study, the clefts observed in their children were all unilateral and out of these 5 children, 3 were males while 2 were females. Though their numbers are too small to make inferences the findings were similar to those of the Tolarova et al. (1990) study where a higher proportion of environmental factors in the aetiology of

clefting resulted in the less severe forms of clefting and usually in the male subset of patients.

Twenty eight (24.8%) of the mothers used prescribed drugs during pregnancy. This was in contrast to the Ghanaian study where use of drugs in pregnancy was not elicited but it was commented that this was indeed a common practice but the respondents may have denied use of the drugs (Donkor et al. 2007). CL was the most common phenotype of these mothers who had used drugs. With respect to laterality, unilateral clefts occurred more commonly than bilateral clefts and this was statistically significant ($p=0.007$). In addition, more males (53.6%) were within this group than females. This was again similar to the findings of the Tolarova et al. (1990) study as pertains the effects of environmental influences to clefting. These findings should, however, be subjected to analytical studies with large sample sizes. Care should also be taken when interpreting results as some of these environmental factors may have been covariates.

Those that reported a positive family history of clefting represented 14% of the families included in the study. This is consistent with the findings in other studies (Osundwa, 2005, Bixler, 1989). The phenotype that was most commonly affected was CL. With respect to laterality, unilateral clefts were more commonly found in this group than bilateral clefts. The children with clefts in this group were also mainly male (56.3%). This finding is contrary to what Tolarova et al. (1990), reports: that when there is a positive family history (heritability), there is a higher proportion of genetic influence and, therefore, the subset of patients more commonly affected are females with bilateral clefts.

The difference in the present findings may be attributed to the small sample. However, it is also possible that the fact that a cleft is familial may not necessarily mean that the genetic influence is higher than the environmental influence. Perhaps these clefts were familial only because this time the mutation is germline and, therefore, can be passed on but the mechanism remains that the mutation increases susceptibility to clefting and only results into clefting when exposed to certain environmental factors. Indeed severity of clefts may be an indication of amount of exposure to harmful environmental risk factors as opposed to an indication of higher genetic influence. There is, therefore, need for a large population study that is able to define environmental and genetic thresholds and interractions in the indigenous African population. It may not be surprising that the findings could be different from those observed in the Caucasian population as studied by Tolarova et al. (1990) since indeed, clefting has racial and geographical variations. Clefting is, therefore, a complex trait and there is need for further investigation to determine the candidate gene mutations in the indigenous African population and how these genes interract with the environment to influence the risk of recurrence.

CONCLUSIONS

The most common phenotype was CL followed closely by CLP while a very low proportion of CP was observed. With respect to laterality, unilateral clefts were the most common. There was no statistically significant difference in the pattern of distribution of clefts among males and females.

The IRF6 gene variant (rs2013162) was found present to a limited extent in this study (2.7% of the children and 2.7% of the mothers) with only one mother transmitting the gene variant to her child. Although an association test could not be carried out, these results seem to suggest that the IRF6 gene variant studied may not be a major genetic influence contributing to non-syndromic clefts in the African population studied. Perhaps a different IRF6 gene variant or different gene altogether may contribute to African non-syndromic clefts.

With respect to the other risk factors, increasing paternal age and prescription drug use in pregnancy (antimalarials and antibiotics) may be important risk factors in the population studied.

MMENDATIONS

1. Further research with a larger population sample should be conducted to test the association between other IRF6 gene variants and non-syndromic clefts in our population.
2. Further research should be conducted to determine other genetic and environmental risk factors that may influence clefting in our population.

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APPENDICES

APPENDIX 1: CLASSIFICATION OF CLEFTS

1. Davies and Ritchie classification (1922): Clefts are divided into 3 groups. Group 1 representing CL, group 2 representing CP and group 3 representing CLP. Groups 1 and 3 are further divide into subsets which describe whether the cleft is unilateral, median or bilateral. Group 2 is subdivided to show the extent of the cleft in the hard and/or soft palate.[24]

CLEFT LIP ALONE	GROUP 1
Unilateral	Subset 1.1
Median	Subset 1.2
Bilateral	Subset 1.3
CLEFT PALATE ALONE	GROUP 2
Soft palate	1/3
	2/3
	3/3
Hard palate	1/3
	2/3
	3/3
CLEFTS OF THE LIPS, ALVEOLUS AND PALATE.	GROUP 3
Unilateral	Subset 3.1
Median	Subset 3.2
Bilateral	Subset 3.3

2. The Veau classification system (1931): This classification system was described in 1931 and classified the degrees of deformity. The system

concentrated mainly on the CP, omitting some CL types for instance, CL and alveolus.[25]

- Group I (A) - Defects of the soft palate only
- Group II (B) - Defects involving the hard palate and soft palate
- Group III (C) - Defects involving the soft palate to the alveolus, usually involving the lip
- Group IV (D) - Complete bilateral clefts

3. Fogh-Anderson Classification (1942): This classification describes the morphology of the cleft based on embryology and genetics. He divided the typical clefts into three main groups then designated atypical clefts as the fourth group.[26]

Harelip (single or double)

Harelip and cleft palate

Cleft palate

Atypical clefts (median, oblique, transverse clefts)

4. Kernahan and Stark classification (1958): This classification highlights the anatomic and embryonic importance of the incisive foramen formed during weeks 4-7 gestational age (GA). The secondary palate forms the roof of the mouth from the incisive foramen to the uvula during weeks 7-12 GA.[27]

This system provides a graphic classification scheme using a Y-configuration, which can be divided into 9 areas.

Areas 1 and 4 - Lip

Areas 2 and 5 - Alveolus

Areas 3 and 6 - Palate between the alveolus and the incisive foramen

Areas 7 and 8 - Hard palate

Area 9 - Soft palate

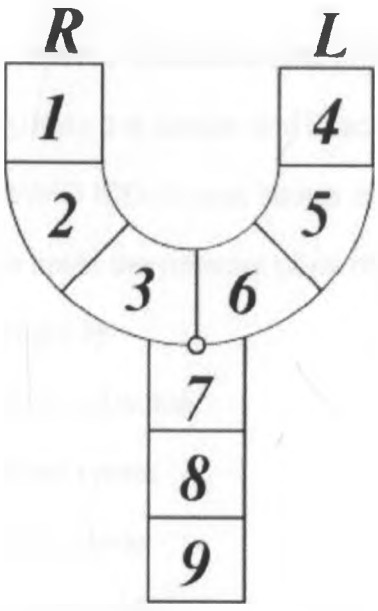

Fig. 9: Kernahan and Stark classification.

5. International Confederation of Plastic and Reconstructive Surgery Classification:

This classification was adopted following the Rome Congress of the International Confederation of Plastic and Reconstructive Surgery in 1967. This system uses an embryonic framework to divide clefts into 3 groups, with further subdivisions to

denote unilateral or bilateral cases. This system is a modification of both the Fogh Anderson and Kernahan and Stark classifications.

Group I - Defects of the lip or alveolus

Group II - Clefts of the secondary palate (hard palate, soft palate, or both)

Group III - Any combination of clefts involving the primary and secondary palates

6. LAHSAL classification (1989): This classification by Kriens was adopted in the UK as it is simple and is accurate for most purposes. It is also compatible with the WHO ICD-10 and allows clefts to be coded for computer use.[28] The LAHSAL code splits the relevant parts of the mouth into six parts:

Right lip

Right alveolus

Hard palate

Soft palate

Left alveolus

Left lip

The upper case letters represent complete clefts while the lower case letters represent incomplete clefts.

7. WHO ICD-10: This is the WHO international statistical classification of diseases and related health services 10th revision, version for 2007. In this classification, typical orofacial clefts are designated letter Q35 to Q37 and broadly classified as isolated CP, cleft lip alone and CLP.

Q35 Cleft palate

Includes: Fissure of palate
palatoschisis

Excludes: cleft palate with cleft lip (Q37.-)

Q35.1 Cleft hard palate

Q35.3 Cleft soft palate

Q35.5 Cleft hard palate with cleft soft palate

Q35.7 Cleft uvula

Q35.9 Cleft palate, unspecified

Q36 Cleft lip

Includes: cheiloschisis
congenital fissure of lip
harelip
labium leporinum

Excludes: cleft lip with cleft palate (Q37.-)

Q36.0 Cleft lip, bilateral

Q36.1 Cleft lip, median

Q36.9 Cleft lip, unilateral

Cleft lip NOS

Q37. Cleft palate with cleft lip

Q37.0 Cleft hard palate with bilateral cleft lip

Q37.1 Cleft hard palate with unilateral cleft lip

Cleft hard palate with cleft lip NOS

Q37.2 Cleft soft palate with bilateral cleft lip

Q37.3 Cleft soft palate with unilateral cleft lip

Cleft soft palate with cleft lip NOS

Q37.4 Cleft hard and soft palate with bilateral cleft lip

Q37.5 Cleft hard and soft palate with unilateral cleft lip

Cleft hard and soft palate with cleft lip NOS

Q37.8 Unspecified cleft palate with bilateral cleft lip

Q37.9 Unspecified cleft palate with unilateral cleft lip

Cleft palate with cleft lip NOS

This study used a modified Fogh Anderson classification. In this classification the typical clefts are divided into three main groups. These were then modified by subdividing to describe the laterality of the cleft as well as the extent of the cleft- whether complete or incomplete. This classification was sufficient to generally describe the phenotype of the clefts as well as their degree of severity.

1. Cleft lip:
- Unilateral
    - Right
        - Complete
        - Incomplete
    - Left
        - Complete
        - Incomplete
- Bilateral
    - Complete
    - Incomplete

2. Cleft lip and palate:
    - Unilateral
        - Right
            - Complete
            - Incomplete
        - Left
            - Complete
            - Incomplete

- Bilateral
    - Complete
    - Incomplete

3. Cleft palate:
    - Unilateral
        - Right
            - Complete
            - Incomplete
        - Left
            - Complete
            - Incomplete
    - Bilateral
        - Complete
        - Incomplete

4. Atypical cleft:
    - Median cleft
    - Oblique facial cleft

APPENDIX 2: CONSENT INFORMATION

Dear **Parent,**

I am a postgraduate student at the University of Nairobi, School of Dental Sciences, pursuing studies leading to specialization in Oral and Maxillofacial Surgery. I wish to request your permission for you and your child to participate in a study that will form part of my degree work. The participation is voluntary and you can withdraw from the study at any stage. This study is on a gene that is thought to cause cleft lip and palate. The results of the study shall be used to determine whether this gene is involved in the condition that your child has and information obtained will help doctors counsel other patients in our setting who are affected by this condition. Information obtained from the study may also eventually help us know preventive measures that can be taken against this condition or contribute to genetic therapy in the future.

The study will involve a clinical examination and for you to answer a few questions to ensure that your child has isolated cleft lip and/or palate. Then cells will be obtained from the inner surface of both parents' cheeks as well as that of the child or from mother and child to obtain DNA which will then be used to examine the gene that is being studied. This gene may be involved in the condition that your child has. You are free to ask any Questions about my study if you require any clarification.

I would therefore appreciate your consent by signing here below.

I, **Dr Emily Nyamu**, confirm that I have explained the relevant parts of the study to the participant.

Signed: _____ Date_____

I, **the participant**, confirm that I have understood the relevant parts of the study and do hereby give consent to participate.

Signed: _____Date_____

Purpose of Study: To determine whether a particular gene (IRF6 gene) which has been found to contribute to the occurrence of isolated cleft lip and palate in other populations, could be present in this condition in our population.

Procedure: It involves clinical examination and answering some questions to ascertain that the patient has non-syndromic cleft lip and palate and then obtaining cells from the inside of the cheek from which DNA samples will be obtained. This is done by rubbing a sterile cotton swab on the inside of the cheek. The questions asked will be on whether there is history of clefting in the family, and on the medical history and history of any medication that the mother could have been on during pregnancy.

Risks: There will be no risks involved because no invasive procedures will be performed and the entire clinical examination will be carried out maintaining absolute hygienic measures.

Benefits: The study will determine whether this gene contributes to the cases of cleft lip and palate. Information from the study and follow-up studies will also help provide options for preventive measures and for treatment in the future (Gene therapy)

Participation: Participation of each child and parent triad or mother child pair is voluntary.

Costs: The entire examination is free.

Confidentiality: No names shall be used in the study therefore the results of the investigation shall be confidential.

Contact of Investigator: Dr. Emily Nyamu.

Dept of Oral and Maxillofacial Surgery

School of Dental Sciences

P.O Box 19676-00202

Nairobi, KENYA.

Cellphone: +254727047000

TAARIFA YA USHIRIKI

Mzazi mpendwa,

Mimi ni mwanafunzi wa shahada ya pili kwenye abara la utabibu na madawa la chuo kikuu cha Nairobi. Nakuomba wewe pamoja na mwanao kushiriki kwenye utafiti wangu. Ushiriki wako ni wa hiari na waweza kujiondoa wakati wowote. Utafiti ni kuhusu kiini kinachoshukiwa kusababisha upasukaji wa mdomo. Utafiti utatumika kubainisha kama upasukaji wa mdomo wa mtoto wako watokana na kiini hicho na matokeo yake yatatumika na madaktari kuwakoga waathiriwa wengine. Matokeo yake huenda yakachangie kupatikana kwa mbinu za kuzuia kuzuka kwake ama hata utibabu katika siku za usoni.

Utafiti utahusu ukaguzi na kujibu kwako maswala kadhaa ili kubainisha kama upasukaji wa mdomo wa mwanao unahusika na magonjwa mengine. Cheche zitazotolewa kwenywe upande wa ndani wa midomo ya wazazi wawili na mwanao zitatumika kwenye utafiti. Waweza kuuliza maelezo kuhusiana na utafiti wangu ili upate kuelewa.

Nitashukuru ukithibitisha hiari yako ya kushiriki kwa kutia sahihi hapa chini.

Mimi, **Daktari Emily Nyamu** nathibitisha ya kwamba nimewaeleza washiriki kuhusu vipengele vinavyohusika kwenye utafiti wangu.

Sahihi: _____Tarehe:_____

Mimi, mhusika, nathibitisha ya kwamba nimeelezwa na nimeridhika kwa maelezo bayana na nakubali kushiriki kwa hiari kwenye utafiti huu.

Jina: _____

Sahihi: _____ Tarehe: _____

Sababu ya utafiti: Kutathmini kama chembechembe aina fulani (IRF6 gene) ambazo zimehusishwa na watu kwingine ndizo zinazozusha kupasuka midomo hapa kwetu.

Muundo msingi: Unahusu uchunguzi wa kidaktari na kujibu maswala fulani halafu kutoa cheche za seli upande wa ndani wa shavu kutumika kwa utafiti. Hizi hutolewa kwa kutumia pamba safi. Maswala haswa ni kuhusu kama kumekuweko na tukio kama hilo la kupasuka mdomo kwenye hiyo jamii na kama kuna madawa fulani mama mzazi alitumia akiwa mja mzito.

Matatizo: Hakuna matatizo yoyote yanayotarajiwa kutokana na utafiti kwani utafanyika kwenye hali ya usafi sanifu.

Mafanikio: Utafiti utathibitisha kama chembechembe hizo zinahusika na upasukaji wa mdomo. Matokeo yatachangia kufwatiliwa kwa muelekeo ili kuepusha visa vingine kuchipuka kwenye siku zijazo.

Kuhusishwa: Kuhusika kwa wazazi wawili na mwana wao ama mama na mwanawe ni kwa hiari.

Gharama: Utafiti wote utakuwa bila malipo.

Kuwekwa siri: Majina ya wahusika wote yatawekwa siri.

Anwani ya Mtafiti: Daktari Emily Nyamu
Department of Oral and Maxillofacial Surgery,
School of Dental Sciences,
P.O Box 19676-00202
Nairobi, KENYA.
Simu ya mkono: +254 727 047000

APPENDIX 3: SCREENING TOOL

QUESTIONNAIRE AND EXAMINATION FORM TO ASSESS NON-SYNDROMIC CASES

1. **Apart from Cleft lip and palate are there any other type of disorders in the family?**

   Yes ☐ **No** ☐

2. **Do you (mother) suffer from any chronic illnesses for which you have been taking or took medication for a long time?**

   Yes ☐ **No** ☐

   If yes, which illness? _____Which medication?_____

3. **Were you on any of the following medication during pregnancy?**

   a. **Phenytoin** ☐
   b. **Warfarin** ☐
   c. **Thalidomide** ☐
   d. **Retinoid** ☐
   e. **Other** ☐ **Please specify _____**

4. **CLINICAL EXAMINATION:**

   Does the patient have any sign of other somatic malformation or any neurological deficit?

   Yes ☐ **No** ☐

APPENDIX 4: DATA COLLECTION SHEET

1. Patient number_____

2. Age of patient_____Birth Order_____

3. Gender of patient: Male ☐ Female ☐

4. Mother's Details:

   Age_____Age at time of child's birth_____

   Education level_____

   Occupation _____

5. Father's Detail

   Age _____Age at time of child's birth_____

6. Did you smoke during pregnancy?

   Yes ☐ No ☐

7. Did you take alcohol during pregnancy?

   Yes ☐ No ☐

8. Is there a family history of clefts in the immediate and/or extended family:

   Yes ☐ No ☐

9. Does anyone else in the immediate family suffer from this condition?

   Yes ☐ No ☐

   If yes, kindly tick affected member(s).

   Mother ☐

   Father ☐

   Siblings ☐ If so how many (excluding the patient) ☐

EXAMINATION CHART

10. Phenotype of cleft

Cleft lip:

- Unilateral
  - Right
    - Complete
    - Incomplete
  - Left
    - Complete
    - Incomplete
- Bilateral
  - Complete
  - Incomplete

Cleft lip and palate:

- Unilateral
  - Right
    - Complete
    - Incomplete
  - Left
    - Complete
    - Incomplete
- Bilateral
  - Complete
  - Incomplete

Cleft palate:

- Unilateral
  - Right
    - Complete
    - Incomplete
  - Left
    - Complete
    - Incomplete
- Bilateral
  - Complete
  - Incomplete

Atypical cleft:

- Median cleft
- Oblique facial cleft

LABORATORY RESULTS

11. Patient's sample

- **Presence of IRF6 gene variation:**

  Yes ☐ **No** ☐

- **Allele 1**_____
- **Allele 2**_____
- **Allele 1 and 2**_____

12. Mother's sample

Presence of IRF6 gene variation:

Yes ☐ **No** ☐

- **Allele 1**_____
- **Allele 2**_____
- **Allele 1 and 2**_____

13. Father's sample:

- **Presence of IRF6 gene variation:**

  Yes ☐ **No** ☐

- **Allele 1**_____
- **Allele 2**_____
- **Allele 1 and 2**_____

APPENDIX 5: SNP GENOTYPING ASSAY CONTENTS

- Sequence-specific forward and reverse primers to amplify the polymorphic sequence of interest.
- Two TaqMan Minor Groove Binder (MGB) probes for distinguishing between the two alleles.
    - Each TaqMan MGB probe contains
        - A reporter dye at the 5′ end of each probe.
            - VIC dye linked to the 5′ end of the Allele 1 probe.
            - FAM dye linked to the 5′ end of the Allele 2 probe.
        - A minor groove binder at the 3′ end of each probe.

A nonfluorescent quencher (NFQ) at the 3′ end of each probe.

APPENDIX 6: ETHICAL APPROVAL

KENYATTA NATIONAL HOSPITAL
Hospital Rd. along, Ngong Rd.
P.O. Box 20723, Nairobi.
Tel: 726300-9
Fax: 725272
Telegrams: MEDSUP", Nairobi.
Email: KNHplan@Ken Healthnet.org

Ref: KNH-ERC/ 01/ 75

24th January 2008

Dr. Emily Nyamu
Dept. of Oral & Maxillofacial Surgery
School of Dental Sciences
University of Nairobi

Dear Dr. Nyamu

RESEARCH PROPOSAL: "IRF6 GENE VARIANTS OF NON-SYNDROMIC CLEFTS OF THE LIP AND/OR PALATE IN A KENYAN POPULATION" (P1/1/2008)

This is to inform you that the Kenyatta National Hospital Ethics and Research Committee has reviewed and **approved** your above cited research proposal for the period 24th January 2008 – 23rd January 2009.

You will be required to request for a renewal of the approval if you intend to continue with the study beyond the deadline given. Clearance for export of biological specimen must also be obtained from KNH-ERC for each batch.

On behalf of the Committee, I wish you fruitful research and look forward to receiving a summary of the research findings upon completion of the study.

This information will form part of database that will be consulted in future when processing related research study so as to minimize chances of study duplication.

Yours sincerely

PROF A N GUANTAI
SECRETARY, KNH-ERC

c.c. Prof. K.M.Bhatt, Chairperson, KNH-ERC
The Deputy Director CS, KNH
The Dean, School of Dental Sciences, UON
The Chairman, Dept. of Oral & Maxillofacial Surgery, UON
Supervisors: Prof. M. Chindia, Dept. of Oral & Maxillofacial Surgery, UON
Dr. Wallace Bulimo, Dept. of Biochemistry, UON
Dr. Loice Gathece, Dept. of Period. & Comm. Dentistry, UON
Dr. Tom Osundwa, Dept. of Oral & Maxillofacial Surgery, UON
Dr. Jeffery C. Murray, University of Iowa, USA

Gertrude's
CHILDREN'S HOSPITAL
Quality Healthcare for Children

08th July 2008

Dr. Emily Nyamu
Registrar, Oral & Maxillofacial Surgery
University of Nairobi
P O Box 41432 - 00100
NAIROBI

Dear Dr. Nyamu

RE: REQUEST TO CONDUCT RESEARCH STUDY

We acknowledge receipt of your request for permission to conduct a study on "IRF6 Gene Variants of Non-Syndromic Clefts of the Lip and/or Palate in a Kenyan population"

The above proposal has been reviewed as entitled and approved by the Standards & Ethics Committee of Gertrude's Hospital thus you can proceed with the study and upon completion submit the research findings for inclusion in our inventory.

We wish you success in your study.

Regards

Dr. Renson Mukhwana
SECRETARY, STANDARDS & ETHICS COMMITTEE

Cc: Dr. V. Indeche — Chair, Standards & Ethics Committee
Dr. A. Laving — Chair, Education & Research Committee
Dr. S. Noah — Hospital Dentist & Smile Train Cordinator

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