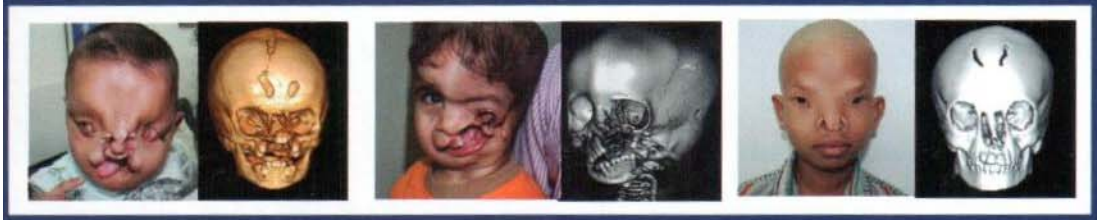
# Management of Cleft & Craniofacial Deformities

Current Techniques, Research & Future Directions

**"Cleft Prevention in Limited Resource Settings"**

## TRANSACTIONS OF
## 7th BIENNIAL WORLD CLEFT LIP & PALATE CONGRESS
*of*
International Cleft Lip and Palate Foundation (ICPF)



May 7-11, 2012

Mahe, Republic of Seychelles

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# Thirty years of studies, experience, and nuts and bolts on our journey toward primary prevention of cleft lip and palate

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## ABSTRACT

In 1982, we published in Lancet our study suggesting that nonsyndromic cleft lip and palate (NCLP) can be prevented by periconceptional supplementation with folic acid (FA). Our studies later showed 65% decrease in recurrences by mother's daily supplementation of multivitamins with 10mg of FA (Teratology 1995) and 27-50 % decrease in occurrence when diet contained 400mcg of FA (Lancet 1995). Several studies that followed confirmed our results, but there were also other that did not support them. **Objective :** To present results of our studies of genetic and environmental factors involved in etiology of NCLP and summarize data from studies by others in order to clarify realistic and effective approach to primary prevention of NCLP in developed and developing countries. **Methods :** This presentation is focused on folate-related genes. We analyzed MTHFR 677CT and RFC1 80AG in 2021 individuals affected with NCLP and 1203 control individuals from 18 locations in 11 countries. We also analyzed maternal nutrition using customized Food Frequency Questionnaire (FFQ) and additional data using General Genetic Questionnaire that we developed. **Results :** We found significant differences in genotype distribution for MTHFR 677CT and RFC1 80AG among populations studied. Analysis of FFQ revealed that four nutrients were associated with NCLP most often: low intake of folate, zinc, and B6 vitamin, and high intake of vitamin A. **Conclusions :** Our studies combined with studies of others show enough scientific evidence that a significant proportion of NCLP can be prevented. However, different genes are creating susceptibility for NCLP and different environmental factors triggering them exist in specific populations: "ONE SIZE DOES NOT FIT ALL". Therefore, prevention approach has to address differences in genetic and environmental factors that exist among different populations. **Acknowledgement :** Rotaplast Intl., Inc. supported field work for our studies and University of the Pacific supported molecular genetics and nutritional analyzes. **Keywords :** Nonsyndromic cleft lip with or without cleft palate, folic acid, primary prevention, MTHFR, NCLP, FA, RFC1, gene polymorphisms.

Figure 1 : Francis Burian

Figure 2 : Lyndon A. Peer

Figure 3 : Richard Smithelles and Marie Tolarova

## INTRODUCTION

### History of nonsyndromic cleft lip and palate prevention and my personal remarks

Several years ago, I recalled in one of my papers a moment that shaped my professional career: "I still remember very vividly that late summer afternoon many years ago at the clinic in the University Hospital in Prague, when I saw for the first time a real baby with a bilateral cleft lip and palate. There was no comparison with all these pictures I remembered from my medical books! His mother was holding him and he smiled at me with his whole face, with his beautiful eyes, chubby cheeks, and -- with a very wide smile. His upper lip was twice broken by clefts. He was of the same age as our baby-boy Martin. Comparing these two babies' faces hurt more than I should have allowed as a professional."[1]. It was in 1964. At that time, I was just a fresh pediatrician looking forward to my medical career to cure, to help heal physical and emotional pain. But at that time I felt hopeless, because I knew that even repairing his cleft would not completely solve his problem. Endless questions - "Why did this happen?," "What did I do wrong?," "Is this going to happen again?, to me?, to my children?", etc. -- that mothers of children affected with a cleft asked me, motivated my professional orientation toward understanding of causes of orofacial clefts and finding ways how to prevent them.

The story continued, the boy and his family became my patients; mother was one of those "case mothers" in the Table 1 who went through periconceptional supplementation and delivered a healthy baby.

Our son Martin and "the boy" and his unaffected brother are mature men and fathers and all have healthy children. And there are many others like that. However, there are still not so few of our patients or parents of a child with a cleft, who were not so lucky - we still had three children affected with NCLP in our cleft prevention study[2-4] (see below).

My interest and work toward prevention of nonsyndromic cleft lip with or without palate (NCLP) started in 1960's. I was strongly influenced by Dr. Francis Burian, Chair of the Department of Plastic Surgery in the University Hospital in Prague, Czech Republic, where I worked. Dr. Burian (Figure 1) returned once from a meeting of plastic surgeons held in San Francisco, California, and shared with us his excitement regarding several attempts and studies done by his

**Table 1** : Czech cleft prevention study - nonrandomized prospective trial of prevention of recurrences by multivitamins and high folic acid (10 mg/day) periconceptional supplementation

| Type of cleft of proband | Controls - non-supplemented | | Cases - supplemented | | Efficacy | |
|---|---|---|---|---|---|---|
| | Without cleft | With cleft | Without cleft | With cleft | Expected | Decreased by(%) |
| NCLP (1) | 1,824 | 77 | 211 | 3 | 8.67 | **65.4** |
| Male with NCLP (2) | 1,149 | 42 | 129 | 1 | 4.58 | 78.2 |
| Female with NCLP (3) | 675 | 35 | 82 | 2 | 4.14 | 51.7 |
| Unilateral NCLP (4) | 1,511 | 55 | 163 | 1 | 5.76 | **82.6** |
| Bilateral NCLP (5) | 313 | 22 | 48 | 2 | 3.29 | 39.2 |

Fisher's exact test was used for all results: (1) P=0.030579; (2) P=0.063169; (3) P=0.227924; (4) P=0.02433612; (5) P=0.3734264

friends - plastic surgeon from New Jersey, Dr. Peers (Figure 2), by Dr Conway, and by Dr Douglas that were presented and discussed at the meeting[5-9]. Soon, Dr Burian initiated a cleft prevention study in Prague[10] and asked me, if I would like to run it.

It was at the 2nd International Congress on Cleft Lip/Palate and Related Craniofacial Anomalies in Copenhagen in 1973, when I the first time publicly mentioned that we were carrying a prospective cleft prevention study using periconceptional supplementation with a high dose of folic acid and multivitamins and shared the first information about our results[11]. There, Dr. Paul Fogh-Andersen, while we were dining in his house, encouraged me to try hard to find out how we could prevent clefts. It was another very strong push and support from this legendary plastic surgeon who published (in the year when I was born!) the first genetic analysis of families with cleft lip and palate[12].

Studies in the US continued[13] and more European colleagues were also searching for the answer whether cleft lip and palate anomalies can be prevented[14-16].

## Our first NCLP prevention results and neural tube defect prevention studies

But not only unanswered questions about etiology of NCLP created curiosity among clinicians and researchers. Another serious and common congenital anomaly severely debilitating child's life - neural tube defects (NTD) - became intensively studied in relation to environmental triggers and specifically to a role of folic acid. The history, however, goes much further into the past. In 1930's and 1940's, the studies on experimental animals indicated that mother's vitamin


**Figure 4** : Composition of our study populations

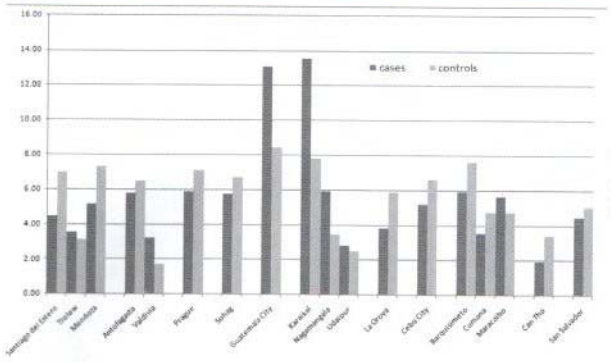


**Figure 5** : Proportion of cases and controls in each subpopulation by city of origin

deficiency could cause congenital malformations in offspring[17-19]. In 1960's, it was demonstrated that formiminoglutamic acid excretion test for defective folate metabolism was more often positive in women pregnant with a child with NTD or other congenital anomaly than in controls[20] and later that periconceptional supplementation with multivitamins[21] or folic acid[22] had a role in prevention of NTD. However, prevention of congenital anomalies seemed impossible to realize as an ultimate goal of teratology[23] and a need for a randomized clinical trial (RCT) was apparent.

I was fortunate to be a close friend and colleague with Dick Smithells (Figure 3) and Michael Laurence. They both encouraged and pushed me to publish the first results of our cleft prevention study in the Lancet in 1982[24]. I remember a nice evening with Michael Laurence and Nicholas Wald in a London pub, where they excitedly discussed their ongoing double-blind, multicenter RCT, supported by the Medical Research Council and how their excitement continued when I showed them my results from the Czech cleft prevention study - they pretty much ordered me to submit it to the Lancet.

We demonstrated a significant decrease of recurrences in the case group of 85 supplemented pregnancies (p=0.023, Fisher exact test). Our study was ongoing and several presentations and publications followed reporting progress of this study[2-3, 25-29].

A randomized, controlled, double-blind, multicenter clinical trial sponsored by the British Medical Research Council (MRC) showed a 72% decrease in the recurrence of NTD when women took 4 mg/day of folic acid periconceptionally[30-32]. Thus, results clearly demonstrated

**Figure 6** : Forme fruste - CL microform on the left

**Figure 7** : Incomplete unilateral left side CL

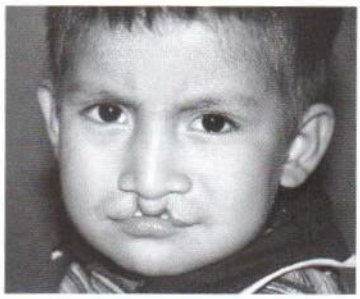

**Figure 8** : Bilateral incomplete cleft lip

the evidence for a role of folic acid (FA) in the prevention of NTD. Moreover, there appeared numerous articles pointing to a preventive effect of FA in other dysraphic congenital birth defects[33-34]. Some also suggested a possible preventive effect of FA in respect to chromosomal aberrations[35].

## Folic acid and prevention of NCLP

A number of observational and interventional studies focused on a preventive effect of FA on orofacial cleft anomalies[4, 24, 33-34, 36-53]. While the vast majority presented suggestions or evidence for a preventive effect, an unequivocal agreement was not found. An extensive overview of these studies was compiled recently by Wehby and Murray[54].

All these findings confirm an urgent need for a randomized, double-blind, controlled multicenter trial to rigorously and conclusively establish whether periconceptional vitamin supple-

**Table 2** : Sample characteristics

| Country | City | Cases | | Controls | | Total | |
|---|---|---|---|---|---|---|---|
| | | n | % | n | % | n | % |
| Argentina | Santiago del Estero | 90 | 4.45 | 84 | 6.98 | 174 | 5.40 |
| | Trelew | 72 | 3.56 | 38 | 3.16 | 110 | 3.41 |
| | Mendoza | 104 | 5.15 | 88 | 7.32 | 192 | 5.96 |
| Chile | Antofagasta | 117 | 5.79 | 78 | 6.48 | 195 | 6.05 |
| | Valdivia | 66 | 3.27 | 21 | 1.75 | 87 | 2.70 |
| Czech Republic | Prague | 119 | 5.89 | 86 | 7.15 | 205 | 6.36 |
| Egypt | Sohag | 116 | 5.74 | 81 | 6.73 | 197 | 6.11 |
| El Salvador | San Salvador | 103 | 5.10 | 61 | 5.07 | 164 | 5.09 |
| Guatemala | Guatemala City | 242 | 11.97 | 102 | 8.48 | 344 | 10.67 |
| India | Karaikal | 274 | 13.56 | 94 | 7.81 | 368 | 11.41 |
| | Nagamangala | 131 | 6.48 | 42 | 3.49 | 173 | 5.37 |
| | Udaipur | 57 | 2.82 | 30 | 2.49 | 87 | 2.70 |
| Peru | La Oroya | 78 | 3.86 | 71 | 5.90 | 149 | 4.62 |
| Philipines | Cebu City | 105 | 5.20 | 80 | 6.65 | 185 | 5.74 |
| Venezuela | Barquisimeto | 120 | 5.94 | 92 | 7.65 | 212 | 6.58 |
| | Cumana | 72 | 3.56 | 57 | 4.74 | 129 | 4.00 |
| | Maracaibo | 115 | 5.69 | 57 | 4.74 | 172 | 5.33 |
| Vietnam | Can Tho | 40 | 1.98 | 41 | 3.41 | 81 | 2.51 |
| TOTAL | | 2021 | 100.00 | 1203 | 100.00 | 3224 | 100.00 |

mentation prevents NCLP and, if it does, whether the effective agent is folic acid, and more what is the effective dose needed. Over the past eight years, Wehby and Murray have developed the Oral Cleft Prevention Program as a double-blinded RCT that has been run in Brazil to estimate the effect of periconceptional supplementation with a high dose of folic acid (4 mg/day) on prevention of NCLP recurrences.

In our non-randomized interventional study, we found a dramatic reduction of cleft recurrences after periconceptional supplementation with multivitamins and a high dose of FA. Our first results were published in Lancet in 1982[24] and the complete final evaluation later[4]. To assess effects of periconceptional multivitamin and folic acid supplementation on recurrence of NCLP, we prospectively evaluated 221 pregnancies of women at risk for a child with NCLP. A ten-step protocol included multivitamin supplementation with SPOFAVIT (vitamins A, B1, B2, B6, C, D3, E, nicotinamide, calcium pathothenicum) and folic acid (10 mg/day) beginning at least two months before a planned conception and continuing for at least three months thereafter. A comparison group comprised 1,901 women at risk for a child with NCLP who received no supplementation and gave birth within the same period as the study group. In the supplemented group, 3 of 214 informative pregnancies ended with infants with NCLP, a 65.4 % decrease (P=0,031, Fisher's exact test); the expected value of 8.67 was calculated based on the incidence of clefts among first-degree relatives in the comparison group (Table 1).

Subset analysis by proband's sex, severity of NCLP, and both variables showed the highest supplementation efficacy in probands with unilateral cleft (82.6 % decrease, P=0.024, Fisher's exact test). No efficacy was observed for female probands with bilateral NCLP. Generally, the efficacy was greater for subgroups with unilateral cleft in comparison with bilateral clefts and for male probands.

Similarly, a large population-based case control study in California demonstrated that periconceptional use of multivitamins, which usually contained 0.4 mg or more of FA, reduced the risk for NCLP by approximately 27-50 %. This was based on data derived from a population-based case-control study on fetuses and live-born infants with orofacial anomalies in the 1987-89 cohort of births in California. Interviews were conducted with 731 (84.7 %) eligible mothers having an infant with NCLP and with 734 (78.2 %) control mothers having non-malformed infants. Results showed a reduced risk of orofacial clefts, if the mother had used multivitamins containing FA during the period starting one month before conception and continuing for two months after conception. Women who used multivitamins containing FA

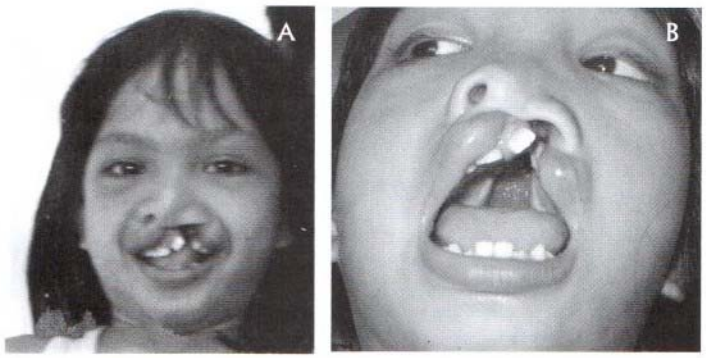


**Figure 9 : A&B** Complete unilateral left side CLP

**Table 3** : Folate-related genes in 18 studied populations. Comparison of proportions of MTHFR 677CT and RFC1 80AG genotypes in cases and controls

| Country | City | Cases | Controls | P value as tested by $\chi^2$ | |
|---|---|---|---|---|---|
| | | n | n | MTHFR 677CT | RFC1 80AG |
| Argentina | Santiago del Estero | 90 | 84 | p=0.050 | p=0.030 |
| | Trelew | 72 | 38 | p=0.041 | p=0.032 |
| | Mendoza | 104 | 88 | p=0.026 | p=0.037 |
| Chile | Antofagasta | 117 | 78 | NS | NS |
| | Valdivia | 66 | 21 | NS | NS |
| Czech Republic | Prague | 119 | 86 | p=0.027 | p=0.038 |
| Egypt | Sohag | 116 | 81 | p=0.048 | p=0.039 |
| El Salvador | San Salvador | 103 | 61 | p=0.028 | p=0.011 |
| Guatemala | Guatemala City | 242 | 102* | p=0.005 | p=0.023 |
| India | Karaikal | 274 | 94 | NS | NS |
| | Nagamangala | 131 | 42 | NS | p=0.049 |
| | Udaipur | 57 | 30 | NS | NS |
| Peru | La Oroya | 78 | 71 | NS | NS |
| Philipines | Cebu City | 105 | 80 | NS | NS |
| Venezuela | Barquisimeto | 120 | 92 | p=0.041 | p=0.032 |
| | Cumana | 72 | 57 | p=0.043 | NS |
| | Maracaibo | 115 | 57 | p=0.037 | p=0.042 |
| Vietnam | Can Tho | 40 | 41 | NS | NS |
| TOTAL | | 2021 | 1101 | NS | NS |

**NS** (non significant) is considered when **p** is higher than 0.05
* additional controls were obtained for continuing ananlysis

periconceptionally had 25-50 % reduction of the risk for an offspring with NCLP compared to women who did not use such multivitamins. However, this association may not be attributable to FA specifically, but may be a consequence of other multivitamin supplement components, or behaviors, that are highly correlated with the use of multivitamins containing folic acid[36].

In 1996, prompted by our published studies that were suggesting preventive effect of FA on recurrence of NCLP[4] and on occurrence of NCLP[36] we organized the symposium "Approach to the Prevention of Orofacial Clefts" in Emeryville, California[55]. Several meetings discussing extensively an optimal design for a NCLP prevention trial followed (WHO Meeting on the Prevention of Orofacial Anomalies in 2001, Global Strategies to Reduce the Health-Care Bur-

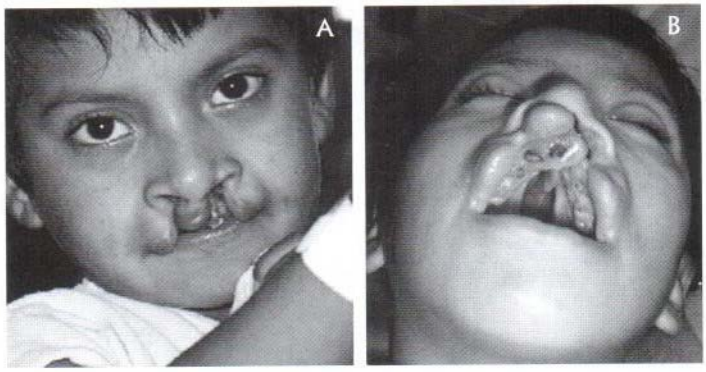


**Figure 10** : **A&B** Complete bilateral CLP

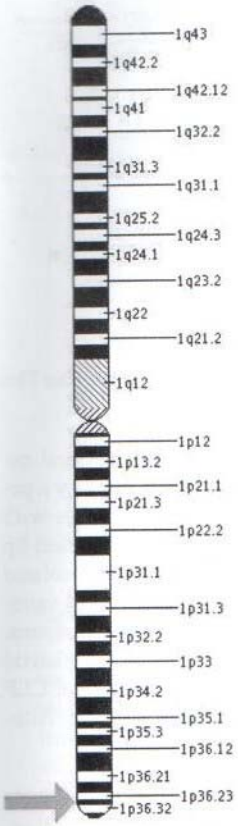


**Figure 11 :** Methylenetetrahydrofolate reductase (MTHFR) gene is located on the short (p) arm of Chromosome 1 at position 36.3

den of Craniofacial Anomalies in 2002). We are aware that there are several key questions that need to be addressed in future scientific studies in order to clarify an association between NCLP and a lack of folate and other vitamins in the maternal diet.

The complex etiology of NCLP in humans is still poorly understood. The vast majority of orofacial clefts (cleft lip, cleft lip and palate, and isolated cleft palate) are nonsyndromic[56-57]. Increasing evidence suggests that NCLP has a multifactorial etiology and both genetics and environment play significant roles in cleft development. It is believed that while genetic factors create susceptibility for clefts, environmental factors trigger development of the cleft through their interactions with a susceptible genotype.

Our long interest in roles of folate metabolism, folic acid supplementation, and folate intake in etiology of NCLP generated our interest in folate-pathway genes. The two most important genes involved in control of the folate metabolism are methylenetetrahydrofolate reductase (MTHFR) and reduced folate carrier 1 (RFC1). We would like to present results of our studies on polymorphisms of these two genes that we studied in 3,224 individuals from 18 populations in 11 countries.

## MATERIALS AND METHODS

### Sample characteristics

Case control study design was chosen for this study. Data and specimens were collected during Rotaplast cleft medical missions in 18 cities in 4 continents between 1998 and 2010. Our study is based on 3224 individuals from 11 countries: Argentina (Santiago del Estero, Trelew, Mendoza); Chile (Antofagasta and Valdivia); Czech Republic, Prague; Egypt, Sohag; El Salvador, San Salvador; Guatemala, Guatemala City; India (Karaikal, Nagamangala, Udaipur); Peru, La Oroya; Philippines , Cebu City; Venezuela

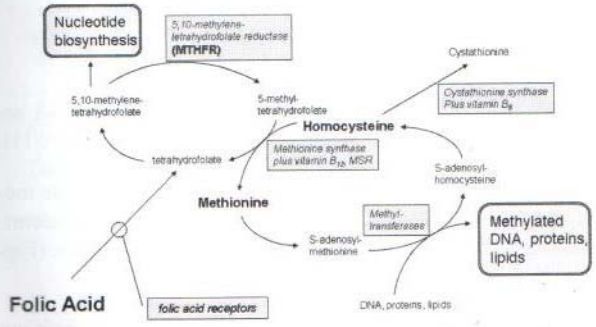


**Figure 12 :** Folate Biochemical pathway (Rosen 1998)

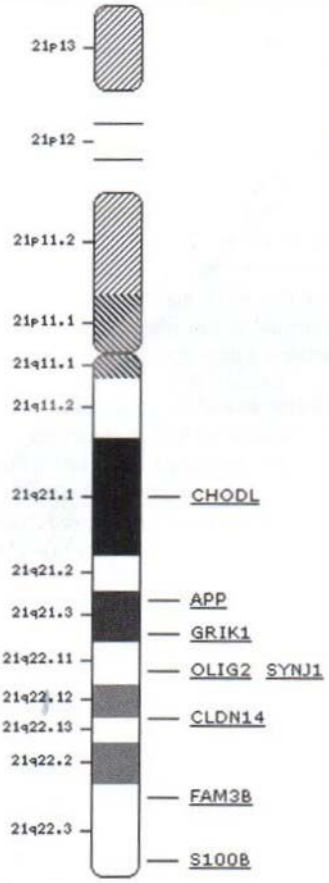


**Figure 13** : Reduced Folate Carrier 1 (RFC1) is located on the long (q) arm chromosome 21 at position 22.3

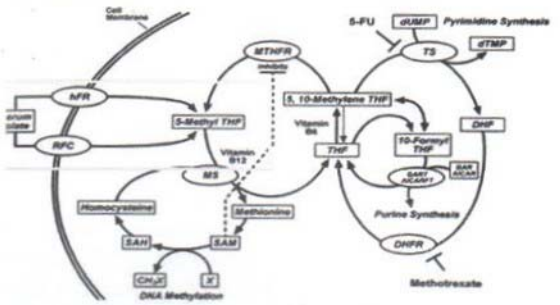


**Figure 14** : Biochemical pathways of RFC1

(Barquisimeto, Cumana, Maracaibo); Vietnam, Can Tho (Table 2, Figures 4 and 5).

Cases and controls were identified by physical examination and by evaluation of medical history by a pediatrician and a medical geneticist. Only cases with nonsyndromic cleft lip (CL) and nonsyndromic cleft lip and palate (CLP) were included, but no cases of isolated cleft palate (CP or CPO = cleft palate alone). All variations of severity of NCLP were specified: microforms, including forme fruste (Figure 6), incomplete unilateral CL (Figure 7), bilateral CL (Figure 8), unilateral CLP (Figure 9a, b), and bilateral CLP (Figure 10a, b). Altogether, 2021 patients form the samples of cases.

The control samples consisted of 1203 individuals with no orofacial cleft, no other congenital anomalies, and no family history of cleft or congenital anomalies in close relatives who originated from the same geographic location. Patients presenting with developmental syndromes were excluded from this study. Table 2 shows proportions of cases and controls from each location studied. Venous blood and/or saliva were obtained for DNA analysis from each individual. One-on-one interview in mother's language was conducted with case and control mothers using the Food Frequency Questionnaire (FFQ) customized for each location as an interview instrument.

## MTHFR gene characteristics

Located on the short arm of chromosome 1 (1p36.3), the MTHFR gene has a cDNA sequence that is 2.2 kilobases long and codes for a catalytically active 77 kDa protein (Figure 11).

5,10-methylenetetrahydrofolate reductase (MTHFR) is an important enzyme in folate metabolism. It converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the predominant circulating form of folate, which is then used to convert homocysteine to methionine (Figure 12).

When the MTHFR enzyme function is altered due to a mutation and this deficiency is not

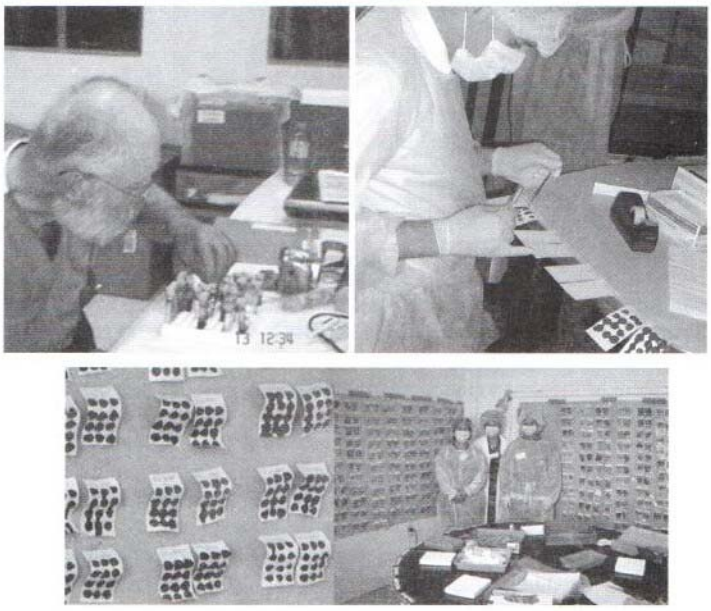

**Figure 15 : A-C** Preparation of blood specimens on site

compensated by an increase in folic acid in the diet, a decrease in metabolically active folate results in a diminished rate of cell multiplication in the early embryonic development and it may lead to an orofacial cleft. MTHFR enzyme is involved in transfer of methyl groups in several important biochemical pathways (Figure 12) including the synthesis of nucleotides, remethylation of homocysteine to methionine and synthesis of proteins, neurotransmitters, phospholipids. A point mutation at the 677 position replaces cytosine (C) with thymine (T) and results in amino acid substitution - alanine is replaced by valine. Homozygous TT genotype forms an impaired thermolabile mthfr enzyme whose capacity to create the 5-methyl form of folate is reduced by 35-50% [58]. Folate in the 5-methyl form is directly involved in the metabolism of nucleotides. As the building blocks of DNA and RNA, nucleotides are essential for multiplication of cells [59]. Normal enzyme function may assist in maintaining the pool of circulating folate, methionine

**Table 4 :** Proportions of MTHFR 677CT genotypes in cases and controls in Guatemalan sample

| Specimens | CC | | CT | | TT | | Total | |
|---|---|---|---|---|---|---|---|---|
| | n | % | n | % | n | % | n | % |
| Cases | 18 | 7.44 | 113 | 46.69 | 111 | 45.87 | 242 | 100.00 |
| Control | 35 | 16.06 | 106 | 48.62 | 77 | 35.32 | 218 | 100.00 |

(□2=10.602; p=0.005)

**Table 5 :** Frequencies of MTHFR C and T alleles in cases and controls in Guatemalan sample

| Specimens | C | | T | | Total | |
|---|---|---|---|---|---|---|
| | n | % | n | % | n | % |
| Cases | 149 | 30.79 | 335 | 69.21 | 484 | 100 |
| Control | 176 | 40.37 | 260 | 59.63 | 436 | 100 |

(÷2=8.802; p=0.003)

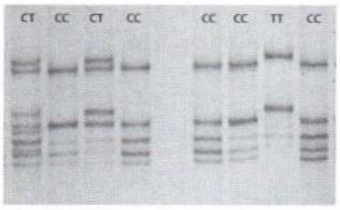


**Figure 16** :PAGE showing homozygous and heterozygous genotypes of MTHFR 677CT

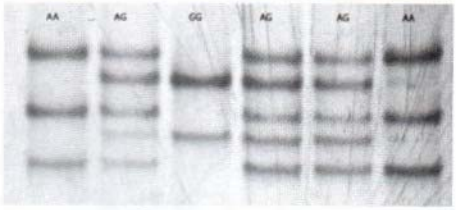


**Figure 17** :PAGE showing homozygous and heterozygous genotypes of RFC1 80AG

and prevent a buildup of homocysteine that may be toxic to embryos and interfere with lip morphogenesis[60]. In those who lack sufficient folate in their diet, a decreased function of mthfr can lead to an increase of homocysteine reaching toxic plasma levels. Given its ability to enter the amniotic fluid of the developing fetus, high levels of homocysteine may be able to disrupt normal palate development through apoptosis induced by oxidative stress[60]. However, there is no consistency of results from studies linking MTHFR 677CT with NCLP.

### RFC1 gene characteristics

The RFC1 gene is located on the chromosome 21 long (q) arm at position 22.3 and it is 27,714 base pairs long (Figure 13).

The gene codes for a protein involved in carrier-mediated transport of folate across the cell membrane. There are several known mutations of the RFC1 gene including the polymorphism at the nucleotide 80 (80AG). The Human Gene nomenclature database symbol is SLC19A1 or solute carrier family 19 folate transport member 1. In the RFC1 80AG polymorphism, the nucleotide adenine (A; wild allele) is replaced by guanine (G; mutated allele) at nucleotide position 80. This missense mutation results in a substitution of glutamine (coded by CAG) with arginine (coded by CGG).

The reduced folate carrier (RFC1) gene encodes a cell membrane transport protein that is necessary for receiving folate molecules from circulating blood and transporting them into cells (Figure 14). Adequate concentration and transport of folate becomes especially important during periods of rapid cell divisions and growth such as infancy and pregnancy. RFC1 also plays a role in transporting folate from endocytotic vesicles into the cytoplasm[61].

**Table 6** : Proportions of RFC1 80AG genotypes in cases and controls in Guatemalan sample

| Specimens | AA | | AG | | GG | | Total | |
|---|---|---|---|---|---|---|---|---|
| | n | % | n | % | n | % | n | % |
| Cases | 35 | 18.61 | 88 | 46.8 | 65 | 34.57 | 188 | 100 |
| Control | 80 | 30.53 | 117 | 44.65 | 74 | 28.24 | 262 | 100 |

($\chi^2$=7.531; p=0.023)

**Table 7** : Frequencies of RFC1 A and G alleles in cases and controls in Guatemalan sample

| Specimens | A | | G | | Total | |
|---|---|---|---|---|---|---|
| | n | % | n | % | n | % |
| Cases | 158 | 42.02 | 218 | 57.98 | 376 | 100 |
| Control | 277 | 53.06 | 265 | 50.77 | 522 | 100 |

($\chi^2$=6.991; p=0.008)

### Collection of specimens

Venous blood was drawn into vacutainer tubes with EDTA from all patients and controls in this study. From some control individuals, saliva specimens were collected. On site, blood spots were made on filter paper with sterile transfer pipettes (Figure 15a, b, c) and allowed to dry for 24-72 hours before being placed in individual envelopes and shipped to the US to the Pacific Craniofacial Genetics Laboratory for DNA analysis.

### DNA analysis

Genomic DNA was extracted from dry blood spots[62]. The fragment of DNA containing the MTHFR 677CT was amplified by polymerase chain reaction (PCR) using specific primers (forward primer : 5'- GTG TGG CAG GTT ACC CCA AA-3'; reverse primer: 5'- TAG CCC TGG ATG GGA AAG AT -3'). For RFC1 80AG, the specific primers (forward primer: 5'- AGCGGTGGAGAAGCAGGT -3'; reverse primer: 5'- GGAGGTAGGGGGTGATGAAG -3') were used.

Genotypes of MTHFR 677CT and RFC1 80AG polymorphisms were identified as specific patterns by a single-strand conformation polymorphism (SSCP) analysis on polyacrylamide gel electrophoresis (PAGE) using a technique similar to that described by Lee[63]. Figure 16 shows patterns indicative for CC, CT, and TT genotypes of MTHFR 677CT and Figure 17 shows patterns indicative for AA, AG and GG genotypes of RFC1 80AG gene polymorphism.

## RESULTS

### Analysis of MTHFR 677CT genotypes

No statistically significant difference in genotype distributions was found when all cases and controls were grouped together and compared. However, as you can see on Table 3, when each location was analyzed separately, proportions of genotypes between cases and controls vary from highly statistically significant (Guatemala City p=0.005, Mendoza p=0.026, Prague p=0.027, San Salvador p=0.028), through significant (Maracaibo p=0.037, Trelew and Barquisimeto p=0.041, Cumana p=0.043, Sohag p=0.048, Santiago del Estero p=0.050) to statistically not significant differences (Antofagasta, Valdivia Karaikal, Nagamangala, Udaipur,

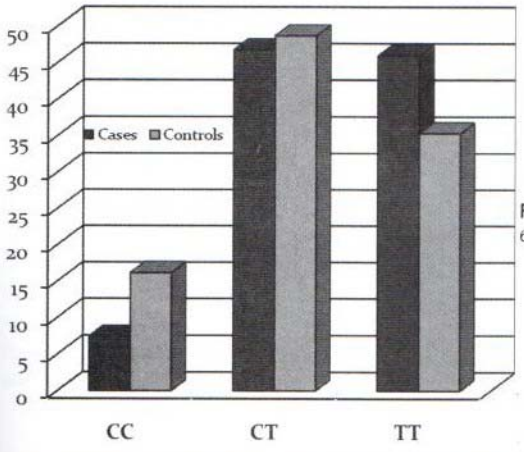


**Figure 18** : Comparison of proportions MTHFR 677CT genotypes in cases and controls

La Oroya, Cebu City, Can Tho). In all cases where statistically significant difference was observed, the higher prevalence of TT homozygotes and in some populations also higher prevalence of CT heterozygotes was found in case samples. Allele frequencies corresponded to genotypes, showing higher T allele frequency in cases in populations with statistically significant difference in genotype distribution.

When each location was analyzed separately, the T allele was significantly more common in cases compared to controls in Guatemala population (p=0.021), followed by Barquisimeto (p=0.041), and Santiago del Estero (p=0.05). As an example, we are presenting analysis of MTHFR 677CT polymorphism in the population from Guatemala City.

### MTHFR 677CT genotypes in Guatemalan population

A significant difference (p=0.005) was found between genotype distributions in cases and controls. In cases, 18 patients (7.44%) had CC genotype, 111 (45.87%) had TT genotype, and 113 (46.69%) were CT heterozygotes. Proportions of genotypes in controls were 35 (16.06%) CC, 77 (35.32%) TT, and 106 (48.62%) CT. Distributions of genotypes at nucleotide 677 of MTHFR gene in cases and controls are shown in Table 4 and Figure 18.

### MTHFR C577T allele frequencies in Guatemalan population

The T allele frequency - as expected based on genotype proportions - was higher in cases than in controls (p=0.003). C allele's frequency was 0.3079 in cases and in controls 0.4037; T allele's frequency was 0.6921 in cases and 0.5963 in controls as shown in Table 5.

### Analysis of RFC1 80AG genotypes

Again, no statistically significant difference was found in RFC1 80AG genotype distributions when all cases were grouped together and all controls were grouped together and then compared (Table 3).

However, similarly as in MTHFR 677CT polymorphism, when each location was evaluated separately, in ten out of eighteen locations (Table 3), statistical significant differences were found (San Salvador p=0.011, Guatemala City p=0.023, Santiago del Estero p=0.30, Barquisimeto and Trelew p=0.032, Mendoza p=0.037, Prague 0.038, Sohag p=0.039, Maracaibo p=0.042,

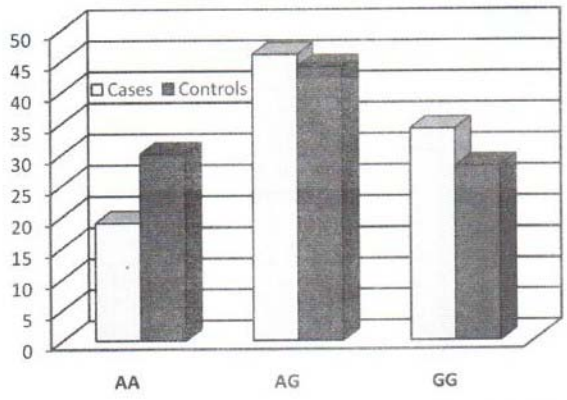


**Figure 19** : Comparison of proportions of RFC1 80AG genotypes in cases and controls

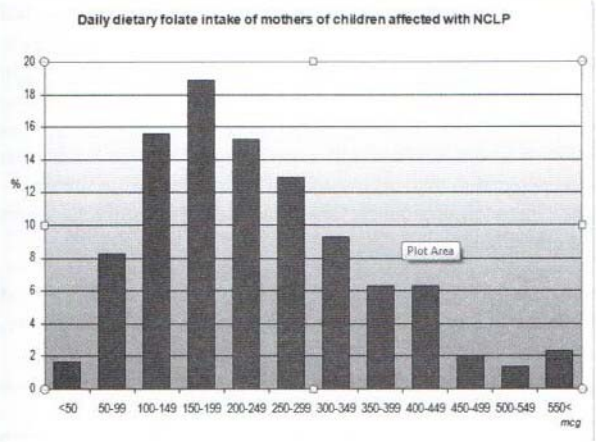


**Figure 20** :Daily dietary folate intake of mothers of children affected with NCLP

Nagamangala p=0.049). Also in RFC1 80AG, higher frequencies of mutated homozygotes GG and of higher heterozygotes AG in some populations were responsible for statistically significant differences in genotype distributions when compared to controls. G allele frequency was higher in cases compared to controls in populations that showed significant differences in proportions of genotypes. Also for RFC1 80AG polymorphism, we are presenting, as an example, our analysis of the population from Guatemala City.

### RFC1 80AG genotypes in Guatemalan population

A significant difference (p=0.023) was found in genotype distribution between cases and controls. In cases, 35 (18.61%) of the individuals had AA genotype, 65 (34.57%) had GG genotype, and 88 (46.80%) had AG genotype. In controls, 80 (30.53%) of the individuals had AA genotype, 74 (28.24%) had GG genotype, and 117 (44.65%) had AG genotype. Distributions of genotypes at nucleotide 80 of RFC1 gene in cases and controls is summarized in Table 6 and Figure 19.

### RFC1 80AG allele frequencies in Guatemalan population

The results for A and G allele proportions in cases and controls are shown in Table 7. Genotype distributions and allele proportions were very similar. The A allele frequency was 0.4202 for cases and 0.5306 for controls, while the G allele frequency was 0.5798 for cases and 0.5077 for controls. The ÷2 test confirmed that there is a significant difference when allele proportions were compared between case and control samples (p=0.008).

### Nutrients in mothers' diet

Studies of environmental factors in etiology of NCLP (among which belongs bioavailability of folate) revealed a special importance of mother's nutrition during prenatal facial development. Data were obtained during 45-60-minute face-to-face interviews of mothers. The interview instruments were the General Questionnaire (medical, pregnancy, and genetic history) and the Food Frequency Questionnaire (FFQ), altogether comprising 152 questions. Using DietSys software, analysis of data from FFQ yielded daily intake of 46 nutrients.

Table 8 summarizes mean values of daily intake of nutrients of case mothers who had a child affected with NCLP. Even when the interview using FFQ was not conducted during pregnancy, we learned that the diet of mothers in countries where we did our study did not vary in time. In other words, the diet of women in those countries was pretty much the same regardless being pregnant or not. Similarly as found for genetic polymorphisms, there were some differences found between daily intakes of nutrients of mothers from different locations. Comparisons with control mothers within each location suggested that environmental factors related to the mothers' nutrition were location specific. Thus, etiological factors associated with orofacial clefts may vary in different locations and ethnicities.

Lower daily intakes of three nutrients - folate, zinc, and B6 vitamin were consistently found in all locations studied. Very high intake of vitamin A was also found in all locations. Therefore,

**Table 8** : Mean values of daily intake of nutrients by mothers of children affected with NCLP Comparison with Recommended Daily Allowance (lower values in red, higher in blue font).

| Nutrient | Case mothers | RDA min | Pregnancy | % of RDA for Women | % of RDA Pregnancy |
|---|---|---|---|---|---|
| Total Calories (cal) | 1572.06 | 2200.00 | 2500.00 | 71.5 | 62.9 |
| Protein (g) | 68.73 | 50.00 | 60.00 | 137.5 | 114.6 |
| Total Fats (g) | 66.03 | N/A | N/A | N/A | N/A |
| Carbohydrates (g) | 182.30 | 130.00 | 175.00 | 140.2 | 104.2 |
| Calcium (mg) | 717.80 | 1000.00 | 1000.00 | 71.8 | 71.8 |
| Phosphorus (mg) | 1127.59 | 700.00 | 700.00 | 161.1 | 161.1 |
| Iron (mg) | 11.81 | 18.00 | 27.00 | 65.6 | 43.7 |
| Sodium (mg) | 3061.76 | 500* | N/A | 612.2 | N/A |
| Potassium (mg) | 2577.50 | 2000.00 | N/A | 128.9 | N/A |
| Vitamin A (RE) | 1163.01 | 700.00 | 770.00 | 166.1 | 151.0 |
| Vitamin A (IU) | 8902.84 | 4000.00 | N/A | 222.6 | N/A |
| Thiamin (mg) | 1.20 | 1.10 | 1.40 | 109.1 | 85.7 |
| Riboflavin (mg) | 1.51 | 1.10 | 1.40 | 137.3 | 107.9 |
| Niacin (mg) | 15.09 | 14.00 | 18.00 | 107.8 | 83.8 |
| Vitamin C (mg) | 88.03 | 75.00 | 85.00 | 117.4 | 103.6 |
| Saturated fat (g) | 22.76 | 1/3 fat | N/A | N/A | N/A |
| Oleic Acid (g) | 23.19 | 1/3 fat | N/A | N/A | N/A |
| Linoleic Acid (g) | 11.10 | 1/3 fat | N/A | N/A | N/A |
| Cholesterol (g) | 374.84 | <300 | N/A | N/A | N/A |
| Dietary Fiber (g) | 14.04 | 20** | N/A | 70.2 | N/A |
| Folate (mcg) | 245.71 | 400.00 | 600.00 | 61.4 | 40.9 |
| Vitamin E (mg) | 7.29 | 15.00 | 15.00 | 48.6 | 48.7 |
| Zinc (mg) | 7.30 | 8.00 | 11.00 | 91.25 | 66.4 |
| Zinc from animal (mg) | 5.66 | 8.00 | 11.00 | 70.7 | 51.5 |
| Vitamin B6 (mg) | 1.16 | 1.30 | 1.90 | 89.2 | 61.1 |
| Magnesium (mg) | 243.27 | 320.00 | 360.00 | 76.0 | 67.6 |
| Retinol (mcg) | 413.85 | 500*** | N/A | 82.8 | N/A |
| alpha-carotene (mcg) | 529.90 | none set | N/A | N/A | N/A |
| beta-carotene (mcg) | 4215.48 | none set | N/A | N/A | N/A |
| Cryptoaxanthin (mcg) | 131.28 | none set | N/A | N/A | N/A |
| Lutein (mcg) | 2272.47 | none set | N/A | N/A | N/A |
| Lycopene (mcg) | 2252.69 | none set | N/A | N/A | N/A |
| Retinol (mcg) | 413.85 | none set | N/A | N/A | N/A |
| Pro-A carotenes (mcg) | 4520.53 | 1500**** | N/A | 301.4 | N/A |

*RDA max 2400 ** RDA max 30*** RDA max 800 **** RDA max 2000
Vitamin A: 1 IU=0.3ug; 4000 IU=1200 ug; 10000 IU=3000 ug

we are proposing to consider these nutrients "candidate nutrients" associated with NCLP.

## Folate

The mean value of daily intake of folate was 245.72 mcg (SD=140.55). This value represents 61.4% of Recommended Daily Allowance (RDA) for women and only 40.9% RDA for pregnant women. The lowest value was 28.8 mcg and the highest value 927.4 mcg. Only 17 mothers out of 302 (11.9%) reported daily intake of folate equal or higher then 400mcg, which is RDA for women. Only 2 mothers reported daily intake of folate higher than RDA for pregnancy (860.6mcg, 927.4 mcg) Figure 20 and 21.

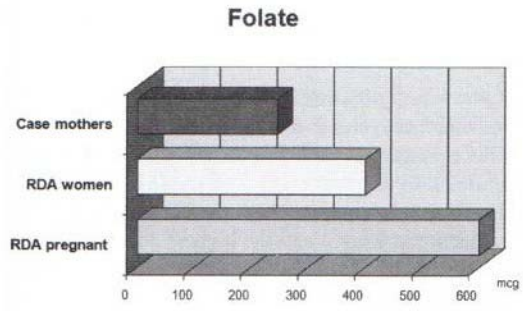


**Figure 21** : Comparison of cases mothers' dietary folate intake with RDA (Recommended Daily Intake) for women and for pregnant women

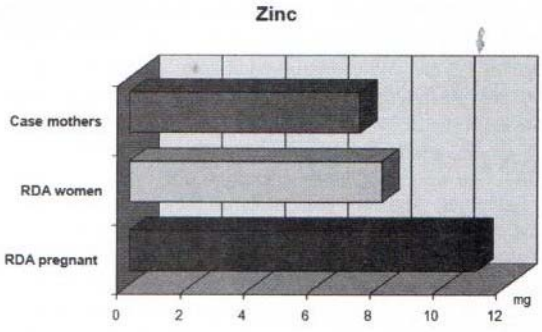


**Figure 22** : Comparison of cases mothers' dietary zinc intake with RDA (Recommended Daily Intake) for women and for pregnant women

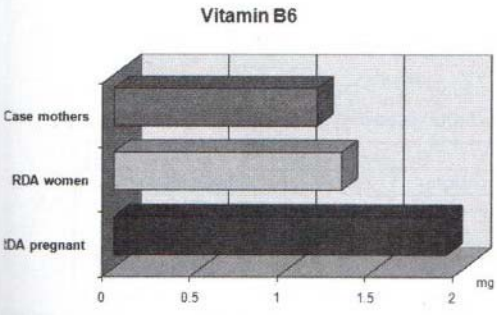


**Figure 23** : Comparison of cases mothers' dietary Vitamin B6 intake with RDA (Recommended Daily Intake) for women and for pregnant women

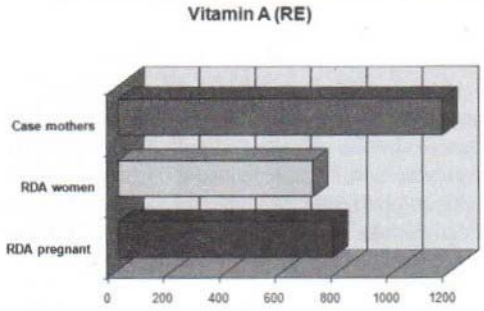


**Figure 24** : Comparison of cases mothers' dietary Vitamin A (RE) intake with RDA (Recommended Daily Intake) for women and for pregnant women

### Zinc

The mean value of daily intake of zinc was 7.3 mg (SD=4.14); the lowest observed value was 2.5 mg and the highest value 25.8. While the mean value is 91.2% of RDA for women, it is only 66.4% of RDA for pregnant women. More precise estimate of intake of zinc is a value of zinc from animal products. The mean value found in our study was 5.66 mg (SD=3.6), that is 70.7% of RDA for women and only 51.45% of RDA for pregnant women. The lowest value of daily dietary intake of zinc from animal products was 0.6 mg and the highest 26.9 mg (Figure 22).

### Vitamin B6

Daily consumption of vitamin B6 was consistently low in all analyzed groups. The mean value 1.16 mg (SD=0.84 represented 89.2% of RDA for women and only 61.05% of RDA for pregnant women. When analyzed in detail, only 30% of women reported daily intake of vitamin B6 equal or higher than 1.9 mg, which was RDA for pregnant women (Figure 23).

### Vitamin A

Extremely high values of daily intake of vitamin A were found in our study. The mean value of vitamin A (IU) was 8902.84 (SD =7404.5) and vitamin A (RE) 1163.01 (SD=814.5). The highest values observed were 69917.4 vitamin A (IU) and 7370.4 vitamin A (RE).

The mean value for vitamin A (IU) represents 222.57% of Recommended Daily Allowance (RDA) for women and the mean value of vitamin A (RE) 166.14% of RDA for women and 151% of RDA for pregnant women (Figure 24).

## DISCUSSION

**Table 9** : Main characteristics of four subgroups of NCLP according to 4-threshold of liability

| Characteristic | Unilateral | | Bilateral | |
|---|---|---|---|---|
| | Male | Female | Male | Female |
| Incidence in general population | 0.0601 | 0.0320 | 0.0188 | 0.0099 |
| Recurrence risk in siblings or children (%) | 2.85 | 4.32 | 6.57 | 8.89 |
| Heritability | 0.6385 | 0.7682 | 0.8749 | 0.8779 |
| Effectivenes of primary prevention* | -2.76 | -2.09 | -1.18 | -0.04 |

*The difference between number of expected and observed cases in treated group

Enormous amount of research effort, time, and funding has been invested into understanding of etiology of NCLP anomalies. Recently, two review articles covered extensively this topic [58-59].

We have known for a long time that orofacial clefts have a significant genetic component[3, 12, 60]. Therefore, individuals affected with a cleft and also their non-affected relatives are at a statistically significantly higher risk to have a child with a cleft compared to the general population. Each individual affected with NCLP has during his/her lifetime 8 to 12 relatives, whose risk for having a child affected with orofacial cleft is 10 to 40 times higher than a risk for clefting in the general population. The highest risk of recurrence - 4% on average (40 times higher than in the general population) - is for the first degree relatives, i.e., for sibs of probands with a cleft and for their children. In other words, at least 4 out of 100 parents who have had one child affected with a cleft or who themselves were born with a cleft, will have a baby with a cleft.

Treatment of children affected with orofacial clefts is challenging, lengthy, and requires multidisciplinary team approach. The average lifetime medical cost for a treatment of one individual with cleft lip/palate in the United States is about $101,000[61]. This includes an immediate cost of $30,000 per patient in the first year of life. In this year alone, the medical cost for babies born with orofacial cleft in the United States will total $750 million.

Prevention, therefore, can save not only suffering, but also millions of dollars! As we know, families with a high risk of recurrence are not only the first on the list of those who need prevention, but they are also the best target population for preventive efforts, because they represent already a selected population in respect to phenotype homogeneity and, therefore, they have the highest probability of a positive effect and the highest return of monetary investment.

We know very well that identification of factors contributing to etiology of NCLP is important for counseling, prevention, treatment planning, and education. We have known for a long time that we need to include also microforms of clefting[62] into this group of congenital anomalies. Recently, not only new microforms were added, but also gene mutations that were associated with them[63-65].

Cleft lip or cleft lip and palate looks the same in a Caucasian, Japanese or Ethiopian baby. It even looks the same, if it is a part of a syndrome. But studies of genes associated with orofacial clefting, even those very sophisticated and expensive genome-wide association studies - GWAS[66-67] have not brought uniform results. As shown in Table 3 - we clearly see that polymorphisms of

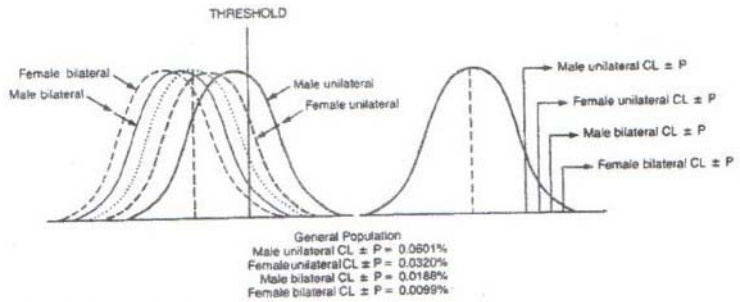


**Figure 25** : Four-threshold model of liability for NCLP

folate-related MTHFR and RFC1 genes have a strong association with NCLP in Central American countries (Guatemala, El Salvador), but no association was found in any of three Indian populations that we studied. One size does not fit all!! Our studies[68] as well as studies by others suggest that different spectra of genetic factors exist in different populations. The same is true about environmental factors, specifically those related to mothers' diet during pregnancy - again, different spectra of environmental factors are found to be associated with NCLP in different populations. In respect to mothers' nutrition, there are some nutrients that seem to be critically important for normal development and we proposed to call them "candidate nutrients". Based on our and studies done by others, these "candidate nutrients" are folate, zinc, and vitamin B6 - when deficient in a diet, and vitamin A - when in a daily intake is higher than normal .

So where is that overlapping area of factors causing NCLP? It is still a mystery. Especially, when considering cleft prevention, we should look at differences in phenotypes. In early 1980's, we introduced a four-threshold model of liability for NCLP[2-3,26,28]. This model shows (Figure 25) that there is a different susceptibility for different types of NCLP according to severity of a malformation and also according to gender of an affected individual. The liability is dependent on a sum of genetic and nongenetic (environmental) factors. It is represented by a curve of normal distribution and a threshold, beyond which individuals are affected with a cleft. Different values of population incidence in males and females subdivide the portion above the threshold into two groups and a similar situation is found when we subdivide the population above threshold by severity of cleft (unilateral or bilateral). Combining both aspects, we developed the


**Figure 26** :Educational brochures about prevention and causes of cleft lip and palate in English, Spanish, Mandarin, Japanese, Korean, and Czech

four-threshold model, in which the highest liability is for females with bilateral clefts, followed by males with bilateral clefts, then males with bilateral clefts an the lowest liability for males with unilateral cleft. The main characteristics distinguishing these four subgroups are presented in Table 9. From the first (male with unilateral CLP) to the fourth (female with bilateral CLP) subgroup, the incidence in general population decreases and the risk of recurrence and the value of heritability increases. Interestingly, results of our method of primary prevention of NCLP (see also Table 1) seem to support our hypothesis of the four-threshold model of liability.

## CONCLUSION

So, where are we with cleft prevention at present? Can we prevent cleft lip and palate? Our answer is YES. There are a significant proportion of NCLP anomalies that are preventable. And if our answer is YES, the next goes HOW? We are not able to fix genetic mutations of these "susceptibility genes", so we need to focus on environmental triggers - and very likely, a balanced maternal nutrition will be most critical. In some populations a folic acid supplementation may be needed, in order to overcome compromised functions of mutated folate-related genes. In other populations it may be other factors or nutrients. What we know for sure is that if we would like to prevent NCLP in a specific location, we need to study the local population first - both genetic and environmental factors.

We have to spread our knowledge on all social levels - education is the first line of battle (Figure 26 - brochures). Collaboration, NOT a competition between disciplines, institutions, clinicians and researchers can make our search for an overlapping area that may be common for the majority if not all NCLP successful.

Francis Collins[69] mentioned in the recent of PBS show that we are in an era of preventive medicine - so let's do it and let's start right now!

## ABBREVIATIONS

FA (folic acid); MRC (Medical Research Council); NCLP (nonsyndromic cleft lip with or without cleft palate); NTD (neural tube defects); RCT (randomized clinical trial)

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