

# Evaluation of homocysteine levels in individuals having nonsyndromic cleft lip with or without palate

Riaz Abdulla, Rouchelle Charmaine Tellis<sup>1</sup>, Roshan Athikari, Jagadish Kudkuli<sup>2</sup>

Department of Oral Pathology and Microbiology, Yenepoya Dental College, Yenepoya University, <sup>1</sup>Department of Microbiology, Yenepoya Medical College, Yenepoya University, <sup>2</sup>Yenepoya Research Centre, Yenepoya University, Deralakatte, Mangalore, Karnataka, India

## Abstract

**Context:** Nonsyndromic cleft lip with or without palate (NSCL  $\pm$  P) is a genetic predisposition involving defects in shape and makeup of the lip and palate. Elevation of homocysteine (Hcy) levels is seen in medical complications such as developmental anomalies causing neural tube defects, congenital vascular diseases, neurodegenerative and psychiatric conditions. Evaluation of serum Hcy levels forms an important feature to look further into molecular aspects.

**Aims:** The aim of this study was to evaluate the Hcy levels in NSCL  $\pm$  P cases by comparing with control cases having no orofacial deformities.

**Settings and Design:** This study was performed with a biochemical assay in a research laboratory.

**Materials and Methods:** A cross-sectional prevalence study was done to compare the concentrations of Hcy between 25 NSCL  $\pm$  P patients and 15 healthy controls. Blood samples were collected from both the patients and controls and assessed for serum Hcy level using competent chemiluminescent immunoassay technique.

**Statistical Analysis Used:** Student's *t*-test was used for statistical analysis.

**Results:** The average Hcy concentration was 9.5  $\mu$ mol/L in control group. There was an increase in Hcy concentration among the NSCL  $\pm$  P cases with an average value of 18.4  $\mu$ mol/L. The results were found to be statistically significant using Student's *t*-test.

**Conclusions:** The results of this study indicate that Hcy concentration has a significant elevation in NSCL  $\pm$  P patients when compared with that of control cases.

**Key Words:** Chemiluminescent immunoassay, homocysteine, nonsyndromic cleft lip with or without palate, orofacial clefts

## Address for correspondence:

Mr. Jagadish Kudkuli, Yenepoya Research Centre, Yenepoya University, Deralakatte, Mangalore - 575 018, Karnataka, India.

E-mail: jagdishk13153@gmail.com

Received: 20.01.2016, Accepted: 29.07.2016

## INTRODUCTION

Nonsyndromic cleft lip with or without palate (NSCL  $\pm$  P) is a genetic predisposition prevalent in the Indian population.<sup>[1]</sup> It involves defects in the shape and makeup of lip and palate

characterized by closure defects of lip, alveolus and/or palate. There have been numerous experimental evidence suggesting the etiology for the disease which gives an approximate frequency of disease incidence in around 1/1000 infants with ethnic and geographic variations.<sup>[2]</sup> Orofacial clefts are one of the

This is an open access article distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 3.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as the author is credited and the new creations are licensed under the identical terms.

For reprints contact: reprints@medknow.com

**How to cite this article:** Abdulla R, Tellis RC, Athikari R, Kudkuli J. Evaluation of homocysteine levels in individuals having nonsyndromic cleft lip with or without palate. J Oral Maxillofac Pathol 2016;20:390-4.

### Access this article online

#### Quick Response Code:



#### Website:

www.jomfp.in

#### DOI:

10.4103/0973-029X.190910

most challenging congenital malformations exhibiting varying phenotype in terms of location of cleft, presence or absence of cleft palate and incomplete fusion of orofacial complex.<sup>[3]</sup> NSCL ± P has a multifactorial etiology including abnormal homocysteine (Hcy) level and anomalous developmental end points which is not well understood.<sup>[4]</sup> The other etiological factors include heredity, consanguinity, maternal environment and demographic factors, and aspects such as intrauterine posture, drugs, vitamins, alcohol consumption, smoking, infections and diet.<sup>[5]</sup> Hence, complexity of the disease gives a scope for research in terms of generating experimental evidence regarding the genetic, environmental and other factors that play a role in causing orofacial defects. Fetal environment can be seen as one of the potential causes of this particular developmental anomaly. Pregnant mothers exposed to nicotine products either by smoking or by indirect ingestion were found to have orofacial malformations in their offspring.<sup>[6]</sup>

The risk of NSCL ± P is expected to be influenced by the patterns of single nucleotide polymorphisms (SNPs), and the resulting variations can have functional consequences ranging from severe to none. As an example, the C677T mutation is considered a risk factor of neural tube defects as it lowered the plasma level of folate.<sup>[7]</sup> Previous studies in NSCL ± P confirmed the role of SNPs in the form of methylene-tetra-hydrofolate reductase (MTHFR) C677T genotype in the mothers who conferred an increased risk of NSCL ± P in their offspring.<sup>[8]</sup> However, there are also reports emphasizing no significant difference in C677T and A1298C polymorphisms between cleft lip/palate (CL/P) patients, their mothers and controls.<sup>[1,3]</sup> These contradicting conclusions at the level of genetic and phenotypic expressions suggest a need for enough evidence to establish exact mechanisms that lead to these developmental anomalies.

One of the main suggested factors responsible for the formation of orofacial clefts is elevated Hcy levels resulting from the errors in Hcy metabolic pathway. Hcy is a sulfur-containing amino acid present in the body which does not take part in the formation of proteins.<sup>[9]</sup> It is formed as an intermediate in the methionine pathway.<sup>[10]</sup> Hcy, under normal conditions, is converted to either methionine by remethylation or to cystathionine through trans-sulfuration pathway [Figure 1].<sup>[11]</sup> The remethylation reaction catalyzed by the enzyme methionine synthase (MS) requires folate and vitamin B12 (or betaine in another type of reaction) as cofactors whereas the trans-sulfuration reaction is catalyzed by cystathionine-β-synthase (CBS) using pyridoxal-5'-phosphate (coenzyme form of vitamin B6) as a cofactor. S-adenosylmethionine allosterically inhibits MTHFR and also activates CBS, thus coordinating the two pathways.<sup>[11]</sup>

MTHFR gene regulates the concentration of Hcy in blood by causing demethylation of 5-methyltetrahydrofolate to

tetrahydrofolate and hence forming methionine from Hcy in the process.<sup>[12]</sup> Any error in the molecular composition of MTHFR gene sequence or gene regulatory mechanism might result in the accumulation of Hcy in blood which might have a role in the formation of orofacial clefts.

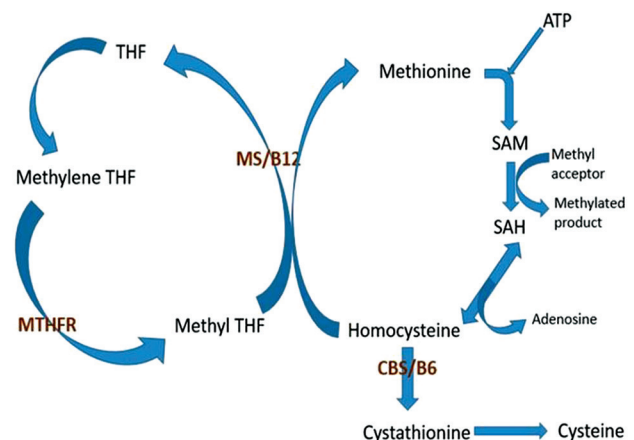
In addition to orofacial cleft formation, disturbed Hcy and folate metabolism is also implicated in many different congenital birth defects such as congenital heart disease, cleft lip, late pregnancy complications, different kinds of neurodegenerative and psychiatric diseases, osteoporosis and cancer.<sup>[13]</sup>

Incidence of NSCL ± P was reported more in males who had orofacial deformities even at relatively lower level of Hcy as compared with females, for whom higher levels of Hcy were required for the deformities to occur. In other words, the incidence of NSCL ± P was more prevalent in males than in females.<sup>[1]</sup>

This study was conducted with the aim of investigating serum Hcy as a risk factor for NSCL ± P. An ideal threshold level of Hcy needs to be established to determine the nutritional deficiencies related to orofacial clefting. Through this study, we confirm the association of elevated levels of Hcy with cleft lip and palate formation with results showing a significant difference in the mean values of Hcy concentration between controls and patients.

## MATERIALS AND METHODS

The study participants were enrolled from the pediatric and general ward of the Hospital. A total of 40 participants were considered, of which 15 were grouped as controls and 25 were grouped as patients. Participants in the control group were the ones not affected with any kind of orofacial deformities. All the cases were evaluated for individual organ system before the



**Figure 1:** Overview of homocysteine metabolic pathway

surgical procedure as per the protocol. There were no associated syndromes or defects in patients as verified by both case record details, physical and other systemic diagnostic examinations. The systemic evaluation was done for cardiovascular system, central nervous system, respiratory system, Ear Nose and Throat and skin conditions. Condition/diseases where Hcy levels were elevated such as congenital vascular diseases, neurodegenerative and psychiatric conditions were excluded in both patient group and control group. This record was obtained from presurgical reports before cheiloplasty surgical procedure. The patient group comprised participants having NSCL ± P with varying degrees of penetrance and phenotypic expressions. The age of participants ranged between 6 and 20 years. Ethical clearance was obtained for the study from the Institutional Ethical Clearance Committee.

Blood samples were collected separately from both control and patient groups and assessed for the concentration of Hcy from serum using competent chemiluminescent immunoassay technology. This is a one-step immunoassay for quantitative determination of total L-Hcy in human serum or plasma. Bound or dimerized Hcy (oxidized form) is reduced by dithiothreitol to free Hcy, which is then converted to S-adenosylhomocysteine (SAH) by the action of the recombinant enzyme SAH hydrolase. The presence of SAH is measured as relative light units (RLUs). There is an inverse relationship between the amount of Hcy present in the patients' sample and the amount of RLUs detected by the system.<sup>[14]</sup>

### Statistical analysis

Student's *t*-test was used to analyze the values of Hcy concentrations obtained in blood samples of both control and patient groups.

### RESULTS

Mean Hcy level in control group samples was  $9.5 \pm 1.99 \mu\text{mol/L}$  and  $18.4 \pm 6.76 \mu\text{mol/L}$  in patient group samples [Figure 2]. *P* value obtained by Student's *t*-test was  $6.84 \times 10^{-6}$ , which is lesser than 0.05. This confirms the significance of the results obtained. Control group comprising individuals without CL/P had relatively moderate level of Hcy in their serum samples [Figure 3] whereas patient group with NSCL ± P had elevated level of Hcy [Figure 4].

### DISCUSSION

The average Hcy level among healthy Indian population is reported to be  $12.5 \mu\text{mol/L}$ . In this study, the average Hcy level was recorded as  $9.5 \pm 1.99 \mu\text{mol/L}$  (mean ± standard deviation) in control group and  $18.4 \pm 6.76 \mu\text{mol/L}$  in patient group. While the former had Hcy levels at relatively healthier range, the latter exhibited elevated levels of Hcy, indicating a possibility of the accumulation of Hcy in blood. Hence, it can

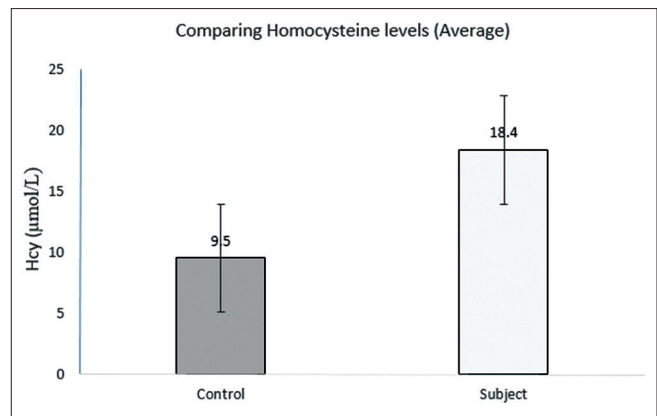


Figure 2: Comparing homocysteine levels in control and patient groups

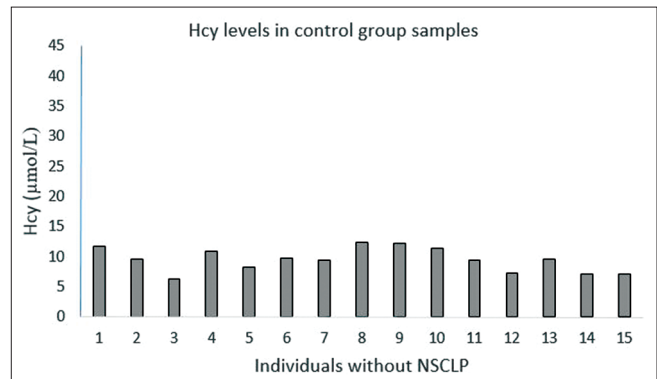


Figure 3: Homocysteine concentration of participants in control group

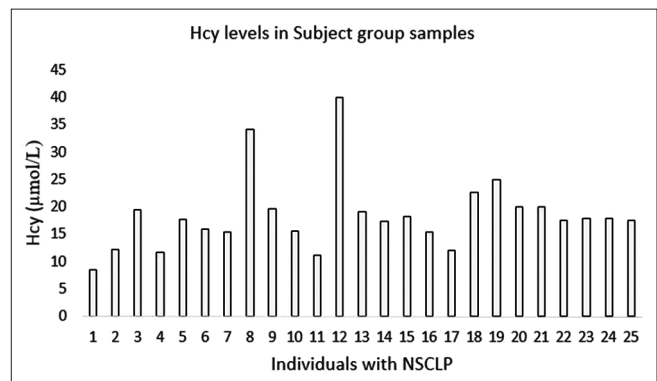


Figure 4: Homocysteine concentration of participants in patient group

be implied that there is a clear increase in the concentration of Hcy in cases having NSCL ± P.

Several intervention and case–control studies have proposed that maternal preconceptional use of multivitamins containing folic acid protects against NSCL ± P occurrence and recurrence.<sup>[15,16]</sup> Folate is a one-carbon donor which is involved in the biosynthesis of purines, pyrimidines and Hcy remethylation, thereby producing methyl groups for methylation of DNA, proteins and lipids. Therefore, folate is important for the expression of several genes essential for cellular multiplication and differentiation during embryogenesis.<sup>[17]</sup> Imbalance in interconversion mechanism of

Hcy and methionine could result in the accumulation of Hcy and lack of free methyl groups that are crucial in post-transcriptional and translational regulation of several genes.

Some have elevated Hcy levels caused by the deficiency of B vitamins and folate in their diets. High Hcy levels are also seen in people with kidney disease, low levels of thyroid hormones, psoriasis and with certain medications (such as antiepileptic drugs and methotrexate). It has been recognized that some people have a common genetic variant (called MTHFR) that impairs their ability to process folate. This defective gene leads to the elevated levels of Hcy in some people who inherit MTHFR variants from both parents.<sup>[18,19]</sup>

The results can also be correlated to the investigations about the compromised supply of myo-inositol, zinc and excessive exposure to glucose which could have a role in the developmental pathogenesis of NSCL ± P. Zinc finger proteins are important in controlling genes in embryonic development. An inadequate zinc status has been postulated to be teratogenic in both animal and human studies. Besides that zinc is involved in the conversion of 5-methyl tetrahydrofolate into tetrahydrofolate by the zinc-dependent MS enzyme.<sup>[20-23]</sup> Both MS and MTHFR enzyme functions in tandem to regulate the amount of Hcy in blood. It was reported that zinc concentration in serum was low in the mothers of neural tube-affected offspring. It should be also noted that NSCL ± P and neural tube defects both arise from neural crest cells.<sup>[24]</sup> It has been shown that Hcy enters the fetus through amniotic fluid and induces apoptosis of the palatal mesenchyme that prevents fusion of the palate.<sup>[25]</sup> Hence, there is a close relationship between zinc concentration and accumulation of Hcy in the blood. Reduction of zinc and folate in the metabolic system can be directly correlated with the accumulation of Hcy in the blood which might act as an important biochemical feature of developmental anomalies in the orofacial region. However, no significant history of maternal folate and zinc status of the investigated cleft children was available for the study.

Determining maternal folate status forms an important parameter in Hcy metabolism since folate-deficient mothers develop a risk of CL/P occurrence or recurrence in their children.<sup>[7,17]</sup>

Therefore, biochemical determination of Hcy compound could act as an indicator to certain molecular mechanism in Hcy metabolic pathway, wherein genes such as MTHFR and MS could play a major role in the development of craniofacial anomalies and act as molecular markers of the disease incidence.

In control samples, there was elevated serum Hcy level in about 2–3 cases. However, it is not of significance since the rise in

value was quite negligible, and ranges characterized for normal, abnormal and moderately elevated Hcy levels are very large.

## CONCLUSION

The genetic basis of NSCL ± P is to be investigated with all possible molecular techniques in the larger Indian cohort. Our study concluded that total serum Hcy concentrations can be a marker for the cleft lip patients. Direct investigation of MTHFR gene polymorphism and Hcy analysis are further being carried out using Sanger sequencing to identify gene variants. Maternal and offspring zinc and folate concentration can also be evaluated as it can influence Hcy accumulation. These studies would establish a link between a group of possible candidate genes and serum concentrations of Hcy, zinc, folate, etc., in the Indian cohort.

## Acknowledgments

This study is supported by the Vision Group Science and Technology (VGST), Government of Karnataka, under the grant no: VGST/SMYSR/GRD-393/2014-15. We are grateful to the funding agency for their valuable support extended for the project.

## Financial support and sponsorship

This study is financially supported by the VGST, Government of Karnataka, under the grant no: VGST/SMYSR/GRD-393/2014-15.

## Conflicts of interest

There are no conflicts of interest.

## REFERENCES

1. Kumari P, Ali A, Sukla KK, Singh SK, Raman R. Lower incidence of nonsyndromic cleft lip with or without cleft palate in females: Is homocysteine a factor? *J Biosci* 2013;38:21-6.
2. IPDTC Working Group. Prevalence at birth of cleft lip with or without cleft palate: Data from the International Perinatal Database of Typical Oral Clefts (IPDTC). *Cleft Palate Craniofac J* 2011;48:66-81.
3. Brandalize AP, Bandinelli E, dos Santos PA, Roisenberg I, Schüler-Faccini L. Evaluation of C677T and A1298C polymorphisms of the MTHFR gene as maternal risk factors for Down syndrome and congenital heart defects. *Am J Med Genet A* 2009;149A: 2080-7.
4. Saikrishna L, Bhaskar LV, Rajasekhar M. Molecular genetics of non-syndromic clefts revisited. *Int J Genet* 2011;3:23-34.
5. Murray JC. Gene/environment causes of cleft lip and/or palate. *Clin Genet* 2002;61:248-56.
6. Lammer EJ, Shaw GM, Iovannisci DM, Van Waes J, Finnell RH. Maternal smoking and the risk of orofacial clefts: Susceptibility with NAT1 and NAT2 polymorphisms. *Epidemiology* 2004;15:150-6.
7. Prescott NJ, Winter RM, Malcolm S. Maternal MTHFR genotype contributes to the risk of non-syndromic cleft lip and palate. *J Med Genet* 2002;39:368-9.
8. Mills JL, Kirke PN, Molloy AM, Burke H, Conley MR, Lee YJ, et al. Methylene tetrahydrofolate reductase thermolabile variant and oral clefts. *Am J Med Genet* 1999;86:71-4.
9. Blom HJ, Smulders Y. Overview of homocysteine and folate metabolism.

- With special references to cardiovascular disease and neural tube defects. *J Inherit Metab Dis* 2011;34:75-81.
10. Rodríguez-Nieto S, Chavarría T, Martínez-Poveda B, Sánchez-Jiménez F, Rodríguez Quesada A, Medina MA. Anti-angiogenic effects of homocysteine on cultured endothelial cells. *Biochem Biophys Res Commun* 2002;293:497-500.
  11. Lentz SR. Mechanisms of homocysteine-induced atherothrombosis. *J Thromb Haemost* 2005;3:1646-54.
  12. Selhub J. Homocysteine metabolism. *Annu Rev Nutr* 1999;19:217-46.
  13. Blom HJ. Folic acid, methylation and neural tube closure in humans. *Birth Defects Res A Clin Mol Teratol* 2009;85:295-302.
  14. Srinivas M, Gupta DK, Rathi SS, Grover JK, Vats V, Sharma JD, *et al.* Association between lower hair zinc levels and neural tube defects. *Indian J Pediatr* 2001;68:519-22.
  15. Homocysteine. ADVIA Centaur Systems: UCSF Clinical Labs-Chemistry [Internet]. Last accessed on 5-08-2016. Available from: [http://labmed.ucsf.edu/labmanual/db/resource/Centaur\\_Homocysteine.pdf](http://labmed.ucsf.edu/labmanual/db/resource/Centaur_Homocysteine.pdf).
  16. Briggs RM. Vitamin supplementation as a possible factor in the incidence of cleft lip/palate deformities in humans. *Clin Plast Surg* 1976;3:647-58.
  17. Tolarova M, Harris J. Reduced recurrence of orofacial clefts after periconceptional supplementation with high-dose folic acid and multivitamins. *Teratology* 1995;51:71-8.
  18. van Rooij IA, Vermeij-Keers C, Kluijtmans LA, Ocké MC, Zielhuis GA, Goorhuis-Brouwer SM, *et al.* Does the interaction between maternal folate intake and the methylenetetrahydrofolate reductase polymorphisms affect the risk of cleft lip with or without cleft palate? *Am J Epidemiol* 2003;157:583-91.
  19. Varga EA, Sturm AC, Misita CP, Moll S. Cardiology patient pages. Homocysteine and MTHFR mutations: Relation to thrombosis and coronary artery disease. *Circulation* 2005;111:e289-93.
  20. Malinow MR, Bostom AG, Krauss RM. Homocysteine, diet, and cardiovascular diseases: A statement for healthcare professionals from the Nutrition Committee, American Heart Association. *Circulation* 1999;99:178-82.
  21. Cavdar AO, Bahçeci M, Akar N, Erten J, Bahçeci G, Babacan E, *et al.* Zinc status in pregnancy and the occurrence of anencephaly in Turkey. *J Trace Elem Electrolytes Health Dis* 1988;2:9-14.
  22. Tamura T, Goldenberg RL. Zinc nutriture and pregnancy outcome. *Nutr Res* 1996;16:39-81.
  23. McDermott A, Gustafsson M, Elsam T, Hui CC, Emerson CP Jr., Borycki AG. Gli2 and Gli3 have redundant and context-dependent function in skeletal muscle formation. *Development* 2005;132:345-57.
  24. Peariso K, Goulding CW, Huang S, Matthews RG, Penner Hahn JE. Characterization of the zinc binding site in methionine synthase enzymes of *Escherichia coli*: The role of zinc in the methylation of homocysteine. *J Am Chem Soc* 1998;120:8410-6.
  25. Krapels IP, van Rooij IA, Ocké MC, van Cleef BA, Kuijpers-Jagtman AM, Steegers-Theunissen RP. Maternal dietary B Vitamin intake, other than folate, and the association with orofacial cleft in the offspring. *Eur J Nutr* 2004;43:7-14.