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## **The Influence of the Role of Health Workers and Health Cadres in Health Education on Changes in Student Behavior Towards Preventing Dengue Fever**

**Rustam Aji Rochmat<sup>1\*</sup>, Dewi Lusiani<sup>2</sup>, Indah Fitri Andini<sup>3</sup>**

**Wiwiek Mulyani<sup>4</sup> Eva Susanti<sup>5</sup> Sherly Ratih Frichesyarius**

**Santi Aji<sup>6</sup> & Roro Ajhie Ayuningtyas<sup>7</sup>**

<sup>1,3,5,\*</sup>Poltekkes Kemenkes Bengkulu.Indonesia,

<sup>2</sup>Poltekkes Kemenkes Jakarta.III.Indonesia,

<sup>4</sup>Poltekkes Kemenkes Jayapura.Indonesia

<sup>5</sup>Puskesmas Curup Timur, Indonesia,

<sup>6</sup>Puskesmas Cibalong.Pamempeuk Garut Kota Jawa Barat.Indonesia

Corresponding Author Email: [adjieroestamadjie@gmail.com](mailto:adjieroestamadjie@gmail.com)

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### **ABSTRACT:**

Dengue hemorrhagic fever (DHF) remains a major public health problem in tropical regions, including Indonesia, due to high morbidity rates and environmental factors that support mosquito breeding. This study aims to determine the influence of the role of health workers and health cadres in health education on changes in student behavior toward dengue fever prevention. Using a single-group pretest-posttest experimental design, 90 respondents were purposively selected and assessed for changes in knowledge and behavior before and after the intervention. The intervention included health education on dengue fever prevention, with a focus on the 3M Plus method: draining, covering, recycling, and using larvicide. The results showed a significant increase in the proportion of respondents with good preventive behaviors, increasing from 15.6% in the pretest to 91.1% in the posttest ( $p\text{-value} = 0.000$ ). This indicates that health education significantly improved community knowledge and encouraged positive behavior changes in dengue prevention. However, sustained community engagement and government support are needed to maintain these improvements. These findings emphasize the importance of structured health education programs in reducing dengue transmission and suggest further research to explore long-term behavioral adherence and its integration with public health policies.

**KEY WORDS:** Role of Health Workers and Health Cadres; Preventive Behavior

### **INTRODUCTION**

Geographically, Indonesia is located between the Asian and Australian continents, the Pacific and Indian Oceans, and is crossed by the equator, giving it a tropical climate. The tropical climate influences factors such as rainfall, temperature, humidity, lighting, and wind, all of which support the development of biota, including disease vectors. In tropical

climates, the development of disease vectors tends to increase, making Indonesia an endemic area for a number of infectious diseases. One of the most common diseases in Indonesia is Dengue Hemorrhagic Fever (DHF), which is caused by the bite of the *Aedes aegypti* mosquito infected with the dengue virus. The disease was first discovered in Surabaya in 1968, with 58 people infected and 24 of them dying. Since then, the disease has spread to various regions, and by 1980, all provinces in Indonesia had DHF (Yuningsih, 2019).

Based on data obtained from the Central Statistics Agency (BPS) of Bengkulu Province, the dengue fever (DHF) case rate in Rejang Lebong Regency has fluctuated in recent years. In 2023, the DHF case rate per 100,000 population was recorded at 102.00. This figure represents a significant increase compared to 2022, which recorded only 36.20 cases per 100,000 population. Previously, in 2021, the DHF case rate in Rejang Lebong reached 127.00, the highest figure in the last three years. Meanwhile, in 2019, the DHF case rate in the area was 114.00.

This fluctuation demonstrates the importance of consistent prevention and control efforts to reduce the DHF case rate in Rejang Lebong Regency. Based on the 2020 report from the Rejang Lebong District Health Office, it was recorded that out of 21 Community Health Centers in Rejang Lebong, especially in Curup, there were three Community Health Centers with the highest number of DHF cases. Perumnas Community Health Center (Puskesmas) was the leading source of dengue fever with 23 cases, followed by Curup Community Health Center (Puskesmas) with 21 cases, and Talang Rimbo Community Health Center (Puskesmas) with 21 cases (Rejang Lebong Health Office, 2020).

The increase in dengue fever cases and the expansion of the affected area over time are due to several factors, including advances in transportation, increasing population density, a lack of public awareness of residential and environmental hygiene, and the spread of the *Aedes aegypti* mosquito, the primary vector of dengue fever, which has spread to almost every corner of the country. Furthermore, the presence of four types of dengue virus circulating throughout the year also contributes to the spread of the disease (Genis, 2016) "National Population and Immunization (PSN) needs to be increased, especially during the rainy and transitional seasons, because increased rainfall can increase the breeding grounds for dengue-transmitting mosquitoes, thus often leading to outbreaks (KLB), especially during the rainy season."

"Mosquito Nest Eradication (PSN) is a crucial effort to control dengue fever (Aji, 2024). Health education is an educational activity aimed at disseminating health messages, strengthening beliefs, and increasing public knowledge, awareness, and understanding so that they are willing and able to make health-related changes. According to Effendy (2012), the goal of health education is to achieve positive behavioral changes in individuals, families, and communities in maintaining healthy behaviors and creating a healthy environment. Furthermore, education also aims to encourage active community participation in efforts to achieve optimal health through the development of healthy behaviors that encompass physical, mental, and social aspects. This is expected to reduce morbidity and mortality (Fabiana, 2019).

Based on research conducted by Takeb & Sabat (2023), the results of counseling and education provided to 66 households in Tunu Village showed that respondents' knowledge was divided into two categories: good and poor. Data collected before (pre) and after (post) counseling showed an increase in the percentage of respondents with good knowledge (pre=40.2%; post=95.4%), good attitudes (pre=42.5%; post=78.2%), and good behavior

(pre=46%; post=52.9%). The results of statistical tests on the knowledge and attitude variables showed a significant difference ( $p < 0.05$ ), while the behavior variable increased from 46% to 60.9%. This indicates that the counseling and education methods have succeeded in increasing knowledge and positive attitudes regarding malaria and mosquito larvae prevention. This increase in positive attitudes is expected to encourage the community to take early preventive measures against mosquito-borne malaria (Takeb & Sabat, 2023) Based on this, the researcher is interested in determining: "The Influence of the Role of Health Workers and Health Cadres in Health Education on Changes in Student Behavior Regarding Dengue Fever Prevention."

## METHOD

This study used a pre-experimental design with a One Group Pretest and Posttest Design, which aimed to measure changes in the experimental group after treatment. The sample used in this study was 90 people selected purposively. The treatment provided was an integration of the 3M Plus movement, which consisted of draining bathtubs, covering water reservoirs, burying used items, and sprinkling abate powder in water reservoirs. Before the treatment was administered, an initial measurement (pretest) was conducted to determine the participants' initial level of behavior regarding preventing vector-borne diseases such as mosquitoes. After the treatment was administered, a final measurement (posttest) was conducted to assess the behavioral changes that occurred in the study participants. The results of the pretest and posttest will be analyzed to determine the effectiveness of implementing the 3M Plus movement in increasing awareness and changing disease prevention behavior.

## RESULTS

Before the health education program, a pretest was conducted to assess participants' initial knowledge. This was followed by a presentation of material covering the definition of dengue fever, symptoms, treatment methods, and preventative measures. Following the presentation, a posttest was administered to assess changes in participants' knowledge. The results of this community service activity are presented in the form of an Influence Index, which compares the average community knowledge score before and after the health education program regarding dengue prevention.

**Table 1. Influence of dengue prevention efforts before and after health education.**

Prevention of dengue	Pretest		Posttest		P-Value
	n	%	n	%	
Good	14	15,6	82	91,1	0,000
Poorly	76	84,4	8	8,9	
Total	90	100	90	100	

Based on the data presented in the table, changes in dengue fever prevention behavior can be seen before and after the counseling. In the pre-test, only 15.6% of participants demonstrated good dengue fever prevention efforts, while 84.4% demonstrated poor prevention efforts. However, after the health counseling (post-test), a significant change occurred, with the number of participants demonstrating good dengue fever prevention efforts increasing sharply to 91.1%, while those demonstrating poor prevention efforts decreased to only 8.9%. This change demonstrates the effectiveness of health counseling in increasing public knowledge and awareness of the importance of dengue fever prevention, particularly in implementing the 3M Plus movement. The statistical test results showed a p-value of 0.000, indicating a significant difference between the pre- and post-conference conditions. Since the p-value is less than 0.05, it can be concluded that health counseling had a significant impact on improving dengue fever prevention efforts in the community.

According to Ali (2010), Health education is an integrated activity within every health effort, aimed at changing individual behavior for healthy living through communication, information, and education. This aligns with Firawan's (2013) research, which states that health education provided to respondents can provide additional information and increase respondents' confidence to behave better in maintaining their health, as also found in research by Reni Ranteallo et al., 2021. The results showed that respondents' knowledge increased after receiving health education provided by the researcher, proving that health education can influence a person's knowledge. This indicates a congruence between existing facts and theory.

## DISCUSSION

Based on the results presented in Table 1, before receiving health education (pre-test), 14 people (15.6%) demonstrated good dengue fever prevention efforts. Factors influencing this included respondents' education level and information received from various sources, such as social media, the internet, and family. Meanwhile, 76 people (84.4%) demonstrated poor dengue fever prevention efforts. This was due to respondents' lack of knowledge about dengue fever prevention methods, due to never having received health education, and laziness, which hindered the adoption of healthy lifestyle behaviors.

After the health education (post-test), the results showed a significant improvement, with 82 (91.1%) respondents now demonstrating good dengue fever prevention efforts, compared to only 14 (15.6%) before the education. This improvement can be explained by respondents' greater understanding of the material presented during the education and increased awareness of healthy living. Furthermore, easy-to-understand counseling materials and the use of appropriate methods also contributed to this change. However, 8 people (8.9%) still Those whose dengue fever prevention efforts have been inadequate, due to a lack of understanding during counseling, limited understanding, and deeply ingrained beliefs and habits that make it difficult to change behavior. Poor residential conditions were also a limiting factor.

In the pre-test phase, data processing showed that not all respondents were able to answer questions about dengue fever prevention efforts correctly. This is understandable, as respondents had not received sufficient information about dengue fever prevention efforts prior to counseling. However, some respondents were still able to provide good answers, as evidenced by the relatively adequate average response rate. This indicates that respondents had received basic information about dengue fever prevention prior to counseling, both through social media and other sources.

These analysis results align with research by Aji (2024). The purpose of this study was to determine the effectiveness of marigold plant powder aroma as a mosquito repellent. The study was conducted in a room containing a mosquito net box with 140 mosquitoes. The results of the Chi-square analysis are as follows:  $P\text{-value} = 0.043 < \alpha 0.05$ , indicating a statistically significant effectiveness between soaking marigold plant root powder in mosquito repellent, with an odds ratio of 4.12 times.  $P\text{-value} = 0.043 < \alpha 0.05$ , indicating a statistically significant effectiveness between soaking marigold plant bark powder in mosquito repellent, with an odds ratio of 4.08 times.  $P\text{-value} = 0.043 < \alpha 0.05$ , indicating a statistically significant effectiveness between soaking marigold flower powder in mosquito repellent, with an odds ratio of 4.06 times.

Students are advised to familiarize themselves with this method by placing soaked powdered roots, bark, and flowers indoors to repel mosquitoes. The results of this analysis align with Kurniawan's (2010) study, "The Effect of Counseling on Community Knowledge Levels and *Aedes aegypti* Mosquito Density in Bayah District, Banten Province". The study showed an increase in community knowledge about Mosquito Nest Eradication (PSN) after the counseling session. However, this increase in knowledge was not accompanied by a decrease in the density and distribution of *Aedes aegypti* mosquitoes. This finding suggests that despite increased public knowledge, the application of this knowledge in practice has not been fully effective in reducing the spread of disease.

This aligns with Notoatmojo's opinion, which states that the influence of knowledge on practice or role can be direct or through attitudes. Knowledge acquired by an individual is not always directly translated into concrete actions. To transform attitudes into concrete actions or practices, supporting factors or enabling conditions are needed, such as environmental support, resources, and stronger individual motivation. Therefore, although counseling can increase knowledge, other factors also need to be considered so that changes in attitudes can be translated into real behavioral changes in disease prevention practices. The results of this study differ from the findings obtained in the study of Dwi Sutakresna and Made Marwati (2020) entitled "General Overview of the Level of Knowledge and Behavior of Heads of Households Regarding the Eradication of Dengue Fever Mosquito Nests (PSN DBD) in the Working Area of the South Kuta Community Health Center".

The results showed that 82 respondents (85.42%) had good behavior in preventing dengue fever by implementing 3M Plus. This finding is in line with the research of Tri Nurul Azizah et al. (2017) entitled "Factors Related to PSN Behavior (3M Plus) as an Effort to Prevent Dengue Fever in the Sendangmulyo Village Community." The results revealed that 51 respondents (56.7%) had good behavior in preventing dengue fever. This difference in results is likely influenced by differences in research methodology, respondent characteristics, or differences in geographical and cultural contexts between research locations. Nevertheless, these two studies have similarities in the implementation of the 3M Plus movement as a dengue fever prevention strategy that has a positive impact on changing community behavior towards health. One factor that can support increased public knowledge is the availability of effective health education. Health education is delivered through various methods, such as counseling, training, and providing technical assistance as needed by the community. Furthermore, the availability of adequate health facilities for individuals, families, and communities is also a crucial factor in supporting increased health knowledge. Adequate facilities can facilitate public access to the necessary health information and services.

Furthermore, the attitudes and behaviors of community leaders and health workers play a crucial role in increasing public knowledge. Health education focused on religious leaders, community leaders, and health workers will be more effective because they are seen as role models in the community. The positive attitudes and behaviors demonstrated by community leaders and health workers can serve as a guide for the community to adopt healthy behaviors in their daily lives. With good examples from community leaders and health workers, the community is more motivated to change their attitudes and behaviors towards a healthy lifestyle. The researchers' assumptions indicate that the majority of the community has demonstrated good behavior in implementing dengue fever prevention through the 3M Plus in their daily lives. This is in accordance with the Stimulus-Organism (SOR) theory, which states that behavioral changes are influenced by the quality of the stimulus interacting with the organism.

To reduce the incidence of dengue fever, active community participation is needed to support the implementation of government programs, as stipulated in Circular Letter Number PM.01.11/Menkes/591/2016 concerning the Implementation of Mosquito Nest Eradication (PSN) 3M Plus with the One House One Mosquito Larvae Movement. Monitoring mosquito larvae and implementing the PSN 3M Plus should be carried out routinely by every family, at least once a week. Unmonitored standing water can become a breeding ground for *Aedes aegypti* mosquitoes, which can lay eggs and spread disease. Therefore, the participation of the entire community in the PSN DHF program is crucial to reducing the risk of transmission of this disease. With active community involvement in prevention efforts, the spread of dengue fever can be significantly minimized, which in turn will contribute to a decrease in the incidence of the disease.

## CONCLUSION

Based on the research results, it can be concluded that health education plays a significant role in improving public understanding and behavior regarding dengue fever (DHF) prevention in remote areas. Before the education, only 15.6% of respondents reported good dengue fever prevention practices, while after the education, this figure increased dramatically to 91.1%. Statistical tests showed a significant difference between before and after counseling, with a p-value of 0.000, indicating the effectiveness of the intervention. Health education, particularly through the 3M Plus program, (drain, cover, recycle, and sprinkle abate) approach, has been shown to increase public awareness of dengue prevention measures. However, challenges remain in translating increased knowledge into long-term behavioral changes, requiring ongoing environmental and policy support. Therefore, collaboration between the community, health workers, and policymakers is needed to strengthen the overall dengue prevention strategy.

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## **A Review on Colorimetric Determinations of Paracetamol by Spectrophotometry**

**D.Chinababu<sup>\*1</sup>, Vishal Bharat Babar<sup>2</sup>, S. Mane Yogita<sup>3</sup>, P. K. Sonali<sup>4</sup>,**

**Y. More Nikita<sup>5</sup>, S.Rakh Shital<sup>6</sup>, S.Shevale Harshala<sup>7</sup>, Sk. Aleesha<sup>81</sup>**

<sup>1-7</sup>Department of Pharmaceutical Analysis and Quality Assurance, DKSS's Institute of  
Pharmaceutical Science and Research (For Girls), Swami-Chincholi, Bhigwan ,  
(Affiliated to Dr.Babasaheb Ambedkar Technological University, Lonere,Pune), Maharashtra, India.

<sup>8</sup>Department of Pharmacology, Dattakala College of Pharmacy, Bhigwan,(Affiliated to Savitribai  
Phule Pune University, Pune), Maharashtra, India.

Corresponding Author Email: [chinababu.rao@gmail.com](mailto:chinababu.rao@gmail.com)

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### **ABSTRACT:**

The colorimetric determinations of Paracetamol in the bulk and pharmaceutical dosage form were carried out with different colouring reagents. The production of a coloured solution served as the foundation for the techniques. The qualitative or quantitative determination is based on the colour formation reactions of Paracetamol with colouring reagents. The wavelengths ( $\lambda_{max}$ ) were used 430,450,468,505,528,606,635,640,650 and 715nm. The calibration curves were constructed from the concentrations ranging from 0.1- 100  $\mu\text{g/mL}$  and regression coefficient values were found to be  $>0.998$ . The % RSD values of the precision were found to be  $<2$ . The methods were more accurate and values were found in between 99-101%. The methods were shown good robustness values and % RSD values were found to be  $<2$ .

**KEY WORDS:** Paracetamol, Colorimetric method, Colouring reagents

### **INTRODUCTION**

Paracetamol (figure-1) is an analgesic and antipyretic<sup>1</sup>. Numerous spectrophotometric techniques have been documented for determining paracetamol, formed the red dye with diazotized 4-nitroaniline (DNAN) in sodium carbonate medium<sup>1</sup>, majority of published methods were on hydrolysis of the compounds leading to the Schiff base formed with a substituted benzaldehyde<sup>2</sup>, reaction of sodium nitrite with drug forms the diazonium salt and coupling reagent was 1-Napthol<sup>3</sup>, 2,4,6-trimethoxybenzaldehyde coupling formed pink colour<sup>2</sup>, the drug forms the colour with p-dimethyl amino benzaldehyde in the 95% ethanol and 2M HCl<sup>5</sup>, estimation of drug in plasma with sodium nitrite, one of the most often

used Cl reagents is luminol<sup>6</sup>. Several oxidizing chemicals, including permanganate ( $\text{MnO}_4^{2-}$ ), oxidize it, hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), periodate ( $\text{IO}_4^{2-}$ ), and hypochlorite ( $\text{ClO}^-$ ).

Luminol undergoes oxidation to yield 3-aminophthalate, an excited state product that emits blue light with a maximum wavelength of 425 nm. Additionally, diperiodatocuprate (III) (DPC), diperiodatoniclate (IV) (DPN), and diperiodatoargentate (III) (DPA) are among the other high and unusual oxidation state transition metal complexes that are being used more and more for analytical purposes<sup>7</sup>. Microwave assisted hydrolysis of paracetamol with 2,2-(1,4-phenylenedivinylene) bis-8-hydroxyquinoline<sup>8</sup>, the colorimetric reagents  $\text{Fe}^{+3}$  and  $\text{K}_3[\text{Fe}(\text{CN})_6]$  were employed for the determination of compound with exhaled breath

condensate technique (EBC), Fe<sup>3+</sup> reduced to Fe<sup>2+</sup> and K<sub>3</sub>[Fe(CN)<sub>6</sub>] chelated the Fe<sup>2+</sup>, oxidative coupling reaction of para amino phenol with p-xylene (2,5-diethylphenol), the process produces a prussian blue (PB) complex by oxidizing paracetamol with Fe(III) and then reacting with ferricyanide in the presence of HCl, charge transfer complex formed between chromogenic reagent of chlorferone with drug, oxidative coupling reaction of paracetamol with phenylephrine hydrochloride in ambient oxygen and in an alkaline media, produce a stable, water-soluble indophenol dye, coupled reaction of 1-naphthol, indirect determination of paracetamol with Luminol-H<sub>2</sub>O<sub>2</sub>-Fe(CN)<sub>6</sub><sup>3-</sup> and based on the oxidation between drug and Fe(CN)<sub>6</sub><sup>3-</sup>, it prevents reaction between luminol and hydrogen peroxide.

The analyte oxidised with K<sub>3</sub>[Fe(CN)<sub>6</sub>] at ambient temperature in ammoniacal aqueous solution by using anionic ion exchange column in the flow injection-spectrophotometric method, alkaline hydrolysis of the analyte formed blue indophenol dye with 8-quinolinol in presence of KIO<sub>4</sub>, reaction with 2-nitroso-1-naphthol-4-sulfonic acid, Stable colour complex produced with a Folin-Ciocalteu reagent, formation of 2-nitro-5-acetamidophenol derivative with nitrous acid in an alkaline medium, intrusion of salicylates and salicylamide in the colorimetric.

Nitration method and grieves the estimation of paracetamol, based on ring-nitration of the drug, using chlorferone, using ZrO<sub>2</sub> (IV) and NH<sub>4</sub>NO<sub>3</sub> (V), Colorimetric method of Glynn and Kendal, reduction of tris(2,2'-bipyridyl) ruthenium(III), luminol-permanganate based reaction, using N, N-dibromo dimethylhydantoin, 4-(Dimethylamino)benzaldehyde react with N-(4-hydroxyphenyl)ethanamide in 2M HCl after heating, fluorophore heroin reagent was used with NaClO oxidizing agent and pH was optimized at 10 with borate buffer.

NaClO oxidizes the analyte and excess of the oxidant determined with o-tolidine dichloride as chromogenic reagent at 430 nm. Paracetamol was dissolved in 4M sulphuric acid and treated with 10.0 mg of sodium bismuthate in the presence of 1M HCl and 1M acetic Acid. It exhibited a stable bluish-violet colour. Paracetamol to p-aminophenol that reacts with S<sub>2</sub>- in the presence of Fe<sup>3+</sup> as oxidant to create a dye that resembles methylene blue and has an  $\lambda_{max}$  at 540 nm. The N-(4-hydroxyphenyl) ethanamide on the hydrolysis formed p-aminophenol and oxidant is dissolved oxygen in the alkaline medium, further formed in to N-acetyl-p-benzoquinone imine, which is capable to produce green indophenol dye with tiron, estimate the drug by using cadmium pentacyanonitrosylferrate modified glassy carbon electrode, Fe<sup>3+</sup> ions oxidize paracetamol and salicylamide when 1,10-phenantroline is present.

## MATERIAL & METHODS

The materials were used in the colorimetric methods paracetamol, diazitized 4-nitroaniline, HCl, benzaldehyde, Na<sub>2</sub>CO<sub>3</sub>, 2,4,6-trimethoxybenzaldehyde, resorcinol, ammonium sulfamate, sodium nitrite, 1-Naphthol, 4,6,4-amino phenol, Na<sub>2</sub>SO<sub>4</sub>, tetrahydroxycalix, indophenol dye, dimethylamino benzaldehyde, chloroacetic acid, NaOH, sulphamic acid, ethanol, luminol, KMnO<sub>4</sub>, H<sub>2</sub>O<sub>2</sub>, KOH, dipiperidodatoargentate (III), K<sub>3</sub>[Fe(CN)<sub>6</sub>], FeCl<sub>3</sub>·6H<sub>2</sub>O, sodium periodate, tylenol, ascarbic acid, phenylephrine hydrochloride, 2,6-dichloro indophenol, n-polyvinylpyrrolidone, styrene, maleic anhydride, benzoyl peroxide, tetrahydro furan, diethyl ether, acetonitrile, poly (styrene-alt-maleic acid), methanol, 2,2-(1,4-phenylene divinylene) bis-8-hydroxyquinoline, ferric chloride, p-xylene (2,5-diethylphenol), potassium ferricyanide, protryliptyline HCl, chlorferone, ceric ammonium sulphate, Iron (III), Zirconium (IV) oxide, ammonium trioxovanadate (V), N,N-Dibromo dimethylhydantoin, O-tolidine, Cadmium Pentacyanonitrosylferrate.

**Preparation of sample solutions:** The majority of published methods were reported the preparation sample solutions depends on acid hydrolysis of the paracetamol with substituted benzaldehyde forms the Schiff base, microwave assisted hydrolysis, few methods have been reported on coupling reactions with coupling reagents, oxidation of the drug with oxidizing agents and oxidation state transition metal complexes and formation of charge transfer complexes.

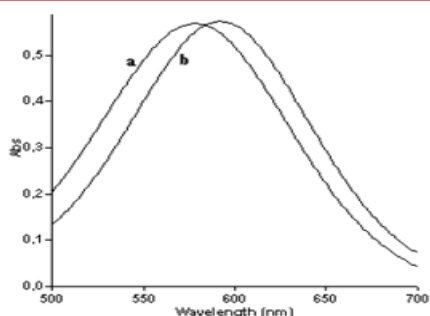
## RESULTS & DISCUSSION

The created procedures were validated in accordance with several recommendations, and the results produced fell within the acceptable range. The reported methods were shown good linearity and regression coefficient values were found to be greater than 0.999. The linearity range were found to be 0.5-20 µg/mL for batch method 1-150 µg/mL for FI method 1, 20-100 µg/mL, 2-10 µg/mL, 1.5-12 µg/mL for conventional UV method for cloud point extraction technique the linearity range reduced to 0.14 - 1.5 µg/mL (figure-2), 1-5 mg/mL, 25-400 mg/L, 0.075-0.75 mg/L, 0.44-5.5 mg/L, 0.2-10 µg/mL, 25-600 µg/mL, 0.01-0.5 µg/mL, 40-160 µg/mL, 50-500 mg/L, 0.5-24 µg/mL, 2-10 µg/mL, 0.1- 2.4 µg/mL, 2-16 mg/mL.

The %RSD of the accuracy obtained results were found to be within the limits as per guidelines. The concentration ranges of the reported methods were found to be from 0-60 mg/ml, 0.8 – 3.36 mL, 0 to 10 µg/ml, 0.01 – 0.70, 1.5—12 mg/mL, 0.075-0.75 mg/L, 0.2–10.0 µg/mL, 2.5-25 µg/mL and >70 µg/mL, 25 to 60 mg/ml, 0.25-30 ppm, 0.2-20 ppm, 0.01-0.5 mg/mL, 10.00–60.00 g/mL, 0.5-24 µg/mL, 2 – 10 µg/mL, 1.0-3.0 mL. The reported values were found the good LOD & the lowest LOD is 0.0369 µg/mL whereas the highest LOD is 40.0 mg/mL. The various methods have been reported and

values were found to be 40.0 mg/mL, 0.0007 mg/mL, 0.2 µg/mL, 0.49 µg/mL, 0.007 µg/mL, 0.0578 µg/mL, 0.02 g/mL, 0.03 g/mL, 0.0448 mg/mL.

**Figure 1: Absorption spectrum of the reaction product with and without Triton-X114**  $\lambda_{\text{max}}$ =580nm ;  $\lambda_{\text{b}}$ = 590nm



The quantification limit values were found to be 0.002 mg/L, 0.166 mg/mL, 0.1753 g/mL, 0.10 g/mL, 0.08 mg/mL, 0.149 mg/mL, 0.121mg/mL. The %RSD values of robustness were found to be within the limits as per guidelines. The review article focused on the colourimetric determination of paracetamol with different colouring reagents and validation parameters. The Hayati filik et al4 reported lowest linearity concentration range from 0.14-1.5 µg/mL with CPE method by using with colourant calixarene (CAL4) in the presence of KIO4 oxidant and colour of the solution was blue. Cloud point phase separation was done with Triton X-114 (surfactant micelles) by addition of Na2SO4. It is the high sensitive method because of detection limit was found to be 40ng/mL.<sup>4</sup>

Paracetamol estimated in urine by using different colouring reagents and their linearity concentration values were reported in table 1.

**Table:1 Colorimetric estimation of Paracetamol in Urine4**

Method	Linearity range (µg/mL)	LOD (ng/mL)	Reference
Phenol	100-800	NR	Welch et al47
o-Cresol	0.3-12	100	Criado et al45
Xylenol	20-400	NR	Chen et al49
Resorcinol	2-100	900	Bocxlaer et al48
8-hydroxyquinoline	0-2.5	NR	Morris et al50
Derivative UV	5-30	2000	Parojicic et al51
Diazotisation	2-10	NR	Heirwegh et al46
ELSD	1-100	300	Criado et al52
UV-Visible	1.5-12	500	Hayati Filik et al4
CPE-UV-Visible	0.14-1.5	40	Hayati Filik et al4

## CONCLUSION

The paracetamol was estimated colorimetrically by using spectrophotometer with different coloring reagents. The charge transfer complex formed between chromogenic reagent of chlorferone with drug and shown sensitivity of the method. Different oxidation reactions were carried with oxidizing (coupling) agents were used with alkaline or acidic medium and produces the more intense colour, determined in visible range. The colourant calixarene (CAL4) in the presence of KIO4 oxidant the drug was produced blue colour. This reagent used for the estimation of drug in the urine, with cloud point phase separation Triton X-114 (surfactant micelles) was used by addition of Na2SO4. It is the high sensitive method because of LOD was found to be 40 ng/mL and linearity range was found to be 0.14-1.5 µg/mL.

The drug produces the stable colored complex with Folin-Ciocalteau reagent it enhance the sensitivity and stability of the method. The other colorants were used benzoyl peroxidepoly (styrene-alt-maleic acid), 2,2-(1,4-phenylene divinylene) bis-8-hydroxyquinoline, ferric chloride, P-xylenol (2,5-diethylphenol), potassium ferricyanide12,16, protryliptyline HCl, chlorferone13,14,31, cerric amonium sulphate13, Iron (III)15, Zirconium (IV) oxide, ammonium trioxovanadate (V)32, N,N-Dibromo dimethylhydantoin36, O-tolidine39, Cadmium Pentacyanonitrosylferrate43.

**Conflict of Interest:** The authors have stated that they don't have any conflicting interests.

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## **Mucocele of the Lower Lip: A Case Series and Review of Surgical Management**

**Dr. Pravin N. Lambade<sup>1</sup>, Dr. Gunjan K. Hiware<sup>2</sup>, Dr. Abhijeet K. Pawar<sup>3</sup>  
& Dr. Samruddhi B. Hatwar<sup>4</sup>**

<sup>1</sup>Academic Qualification: Professor, M.D.S Affiliation: Oral and Maxillofacial Surgery  
Dr. Rajesh R. Kambe Dental College and Hospital, Akola, Maharashtra 444401 India

<sup>2</sup>Academic Qualification: M.D.S Affiliation: Oral and Maxillofacial Surgery  
Dr. Rajesh R. Kambe Dental College and Hospital, Akola Maharashtra 444401 India

<sup>3</sup>Academic Qualification: M.D.S Affiliation: Oral and Maxillofacial Surgery  
Dr. Rajesh R. Kambe Dental College and Hospital, Akola Maharashtra 444401 India

<sup>4</sup>Academic Qualification: M.D.S Affiliation: Oral and Maxillofacial Surgery  
Institute: Dr. Rajesh R. Kambe Dental College and Hospital, Akola Maharashtra 444401  
Corresponding Author Email: [drpravinlambade@gmail.com](mailto:drpravinlambade@gmail.com)

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### **ABSTRACT:**

Mucoceles are common benign lesions of the oral cavity, most frequently affecting the lower lip due to trauma or habitual behaviors such as lip biting. They present as painless, dome-shaped swellings that may vary in size and color, and although benign, can cause discomfort or aesthetic concerns. This case series reports five patients, aged 16 to 52 years, all presenting with lower lip mucoceles of varying durations, sizes, and clinical features. Clinical examination revealed soft, fluctuant swellings, with two cases associated with habitual lip biting and two showing intermittent size fluctuations. All patients underwent complete surgical excision under local anesthesia, including removal of the associated minor salivary glands, followed by histopathological confirmation. Follow-up periods ranged from three months to one year, with no recurrences observed. These findings reinforce the role of meticulous surgical excision and elimination of etiological factors in preventing recurrence and ensuring favorable long-term outcomes.

**KEY WORDS:** Mucocele; lower lip; minor salivary gland; surgical excision; recurrence

### **INTRODUCTION**

Mucoceles are common benign lesions of the oral cavity that result from the accumulation of mucus within the connective tissue due to rupture or blockage of the excretory duct of a minor salivary gland.[1] They are typically caused by mechanical trauma, such as lip biting or direct injury, leading to extravasation or retention of mucus.[2] Clinically, mucoceles present as painless, dome-shaped swellings, often with a bluish or translucent hue, and are most frequently located on the lower lip, although they can occur in other areas of the oral mucosa containing minor salivary glands.[1-3] Their size can vary

from a few millimeters to several centimeters, and they may fluctuate over time due to repeated rupture and refilling.[2,4]

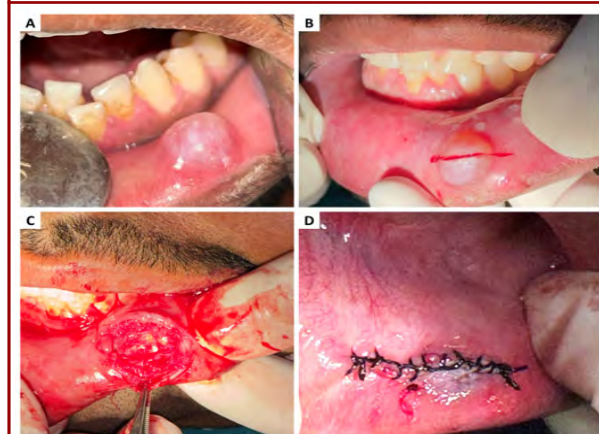
The etiology of mucoceles is multifactorial, with trauma, habitual behaviors, and anatomical predisposition playing key roles.[1-4] While the lesions are benign, they can interfere with oral function, speech, or aesthetics, particularly when large or recurrent.[2-4] Differential diagnosis includes fibroma, hemangioma, lipoma, and salivary gland neoplasms, underscoring the importance of accurate clinical assessment supported by histopathological confirmation.[5]

Management options range from conservative observation,

in cases where spontaneous regression is possible, to various surgical interventions, including complete excision with removal of the associated minor salivary gland to minimize recurrence risk.[6] Alternative methods, such as laser excision, cryosurgery, and marsupialization, have also been reported, each with specific advantages and limitations.[7]. The present case series describes the clinical presentation, diagnostic considerations, surgical management, and follow-up outcomes of mucoceles in five patients. The objective of the case series is to determine common patterns and emphasize the role of complete excision in preventing recurrence.

### Case Presentations:

**Figure 1: Case I – A) Lesion on lower lip, B) Incision, C) Excision of lesion, D) Suturing**

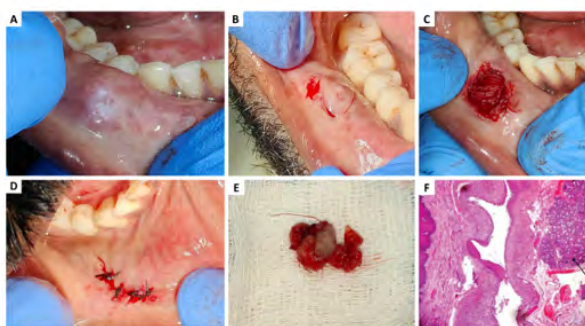


A 24-year-old male presented with a painless swelling of the lower lip of 20–25 days duration (Figure 1). He recalled biting his lower lip about 20 days earlier with subsequent bleeding, after which the lesion gradually enlarged to its present size. There was no history of fever or purulent discharge. Past medical history was non-contributory; he had previously visited a private clinic for the same complaint and received medications. He reported no deleterious habits. On general examination, he was conscious, cooperative, and oriented, afebrile, with pulse 74/min, respirations 16/min, and blood pressure 120/80 mmHg. Extraorally, the facial profile was straight with competent lips, no gross asymmetry, and no palpable lymphadenopathy. Intraorally, a single round-to-oval, soft, fluctuant swelling measuring approximately  $0.5 \times 0.5$  cm was noted on the inner aspect of the lower lip in the 32–33 region, situated 1–2 mm below the vermilion border and contacting the incisal edges of 22 and 63. Additional findings included mild lower anterior crowding, bilateral Angle Class I molar relation, a missing permanent maxillary left canine, and an over-retained deciduous maxillary left canine (63).

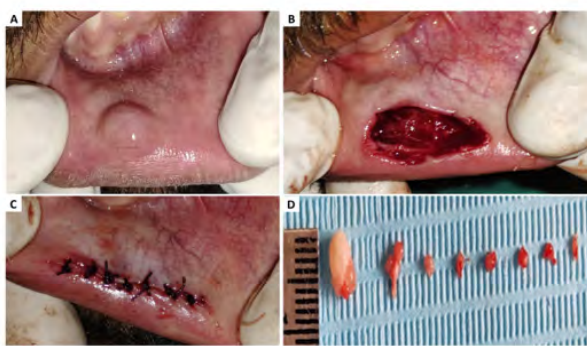
A 52-year-old male presented with a primary complaint of a painless swelling on the inner side of his lower lip on the right side, first noticed three weeks earlier (Figure 2). The swelling had initially been small but gradually increased to its present size.

His medical history was non-contributory. The patient reported a habitual lip-biting behavior, and there was no impairment of speech or mastication. Intraoral examination revealed a single, round, soft, and fluctuant swelling located on the inner surface of the lower lip near the region of the right canine. The lesion was positioned approximately 2–3 mm below the vermilion border and extended downward toward the lingual vestibule, measuring about 6–8 mm in diameter. The overlying mucosa was of the same color as the adjacent tissue, and no other abnormalities were noted in the oral cavity. Based on the clinical presentation and history, a provisional diagnosis of mucocele was considered.

**Figure 2: Case II – A) Lesion on lower lip, B) Incision, C) Excision of the lesion, D) Suturing, E) Excised lesion, F) Pooled areas of mucin (H and E, 4x)**



**Figure 3: Case III – A) Lesion on lower lip, B) Wound after excision, C) Suturing, D) Excised tissue**



A 35-year-old male presented with a complaint of swelling on the lower lip, first noticed approximately three months earlier (Figure 3). The lesion exhibited intermittent changes in size over this period and was not associated with pain. The patient had no relevant medical or systemic history. Clinical examination revealed a soft, painless nodule located on the right side of the labial mucosa. The overlying mucosa appeared normal, and no other intraoral abnormalities were observed. Based on the clinical appearance and history, a provisional diagnosis of mucocele was made.

A 16-year-old male presented with a swelling on the right side of the lower lip, present for about one month, with gradual increase in size over the past three weeks (Figure 4). The lesion

was painless, and the patient admitted to habitual lip biting, particularly during periods of concentration. His medical, surgical, dental, and drug histories were unremarkable, and systemic examination—including neurological, musculoskeletal, respiratory, gastrointestinal, and cardiovascular systems—showed no abnormalities. He denied fever, night sweats, fatigue, weight or appetite changes. Head and neck evaluation revealed no lymphadenopathy, masses, or tenderness, and extraoral assessment showed normal facial symmetry and temporomandibular joint function. Intraorally, a well-circumscribed, soft, dome-shaped swelling measuring approximately  $1.5 \times 1.5$  cm was observed on the right lower labial mucosa adjacent to the occlusal level of teeth 32 and 33. The lesion was mobile, not fixed to underlying structures, and exhibited a bluish hue with a translucent, pearly surface. The overlying mucosa was intact without ulceration or secondary infection. Based on its clinical features and location, a provisional diagnosis of mucocele was made.

**Figure 4: Case IV – A) Lesion on lower lip, B) Incision over the mucosa, C) Excision of the lesion, D) Suturing**



A 23-year-old female presented with a swelling on the lower lip, persisting for the past month (Figure 5). The lesion was soft in consistency, oval in shape, sessile, painless, and demonstrated intermittent variations in size. The patient reported a history of trauma to the lower lip prior to onset. Medical and systemic histories were non-contributory. Intraoral examination revealed a localized swelling on the labial mucosa of the lower lip, with intact overlying mucosa and no signs of ulceration or secondary infection. Based on the clinical features and history, a provisional diagnosis of mucocele was considered.

**Management of the cases:** In all cases, surgical excision of the lesion was performed under local anesthesia. Following aseptic preparation of the operative site, local infiltration was administered using 2% lignocaine hydrochloride with adrenaline in a 1:80,000 ratio to achieve adequate anesthesia and hemostasis. An elliptical incision was made around the lesion using a No. 15C blade, ensuring inclusion of the overlying mucosa and underlying minor salivary gland tissue. Blunt dissection and stripping with a hemostat were carried out to separate the lesion from surrounding tissues, taking care to avoid rupture of the cystic cavity.

The mucocele, along with the associated minor salivary glands, was completely excised to minimize the risk of recurrence. Flap approximation and primary closure were achieved with simple interrupted sutures using 3-0 silk. Postoperative instructions were provided to all patients, including maintenance of oral hygiene, dietary modifications, and avoidance of lip-biting habits. Appropriate medications, including analgesics and antibiotics, were prescribed. Sutures were removed after one week, and follow-up evaluations were scheduled to monitor healing and assess for any recurrence. The details of all cases, along with the outcomes, are summarized in Table 1.

**Figure 5: Case V – A) Lesion on lower lip, B) Incision over the mucosa, C) Excision of the lesion, D) Suturing, E) Healed mucosa**



## DISCUSSION

Mucoceleles are among the most frequently encountered benign lesions of the oral cavity, most often affecting the lower lip due to its susceptibility to trauma and the high concentration of minor salivary glands in this region.[1-4] The pathogenesis is generally related to either extravasation of mucus following ductal rupture or retention of saliva due to ductal obstruction.[8] In the present series, all five cases involved the lower lip, consistent with earlier reports highlighting its predilection site. Habitual lip biting was identified in two cases, supporting trauma as a significant etiological factor.[9]

Clinically, mucoceleles typically present as painless, fluctuant swellings with a translucent or bluish hue, although deeper lesions may exhibit a normal mucosal color.[10] In our cases, lesion size ranged from 0.5 cm to 1.5 cm, aligning with dimensions reported in previous studies, where mucoceleles are generally under 2 cm in diameter. [11] The chronicity varied, with some lesions present for weeks and others persisting for months, and two cases demonstrated intermittent size fluctuations, a feature attributed to periodic rupture and reaccumulation of mucus.[12]

Surgical excision remains the treatment of choice for mucoceleles, as it ensures removal of both the lesion and the affected minor salivary glands, reducing the likelihood of recurrence.[13] All

cases in this series underwent complete surgical excision using an elliptical incision, with careful dissection to prevent rupture during removal. Histopathological examination in each case confirmed

the diagnosis, underscoring its importance in differentiating mucocoeles from other swellings such as fibromas, vascular lesions, or salivary gland tumors.

<b>Table 1. Summary of outcomes of the cases presented in the case series</b>				
<b>Case No.</b>	<b>Age/Sex</b>	<b>Site of Lesion</b>	<b>Treatment Performed</b>	<b>Outcome / Follow-up</b>
1	24/M	Inner aspect of lower lip, 32–33 region	Surgical excision under local anesthesia (2% lignocaine with 1:80,000 adrenaline), elliptical incision, blunt dissection, complete removal with associated minor salivary glands, 3-0 silk closure	Uneventful healing, no recurrence at 6-month follow-up
2	52/M	Inner surface of lower lip, right canine region	Surgical excision under local anesthesia, elliptical incision, blunt dissection, complete removal with associated minor salivary glands, 3-0 silk closure	Uneventful healing, no recurrence at 6-month follow-up
3	35/M	Right side of labial mucosa, lower lip	Surgical excision under local anesthesia, elliptical incision, blunt dissection, complete removal with associated minor salivary glands, 3-0 silk closure	Uneventful healing, no recurrence at 1-year follow-up
4	16/M	Right lower labial mucosa, adjacent to teeth 32–33	Surgical excision under local anesthesia, elliptical incision, blunt dissection, complete removal with associated minor salivary glands, 3-0 silk closure	Uneventful healing, no recurrence at 9-month follow-up
5	23/F	Lower lip, labial mucosa	Surgical excision under local anesthesia, elliptical incision, blunt dissection, complete removal with associated minor salivary glands, 3-0 silk closure	Uneventful healing, no recurrence at 3-month follow-up

Recurrence is a well-documented concern, particularly if the associated minor salivary glands are not completely excised or if postoperative trauma reinitiates the cycle.[14] No recurrences were noted during follow-up periods ranging from three months to one year in our series, suggesting that meticulous surgical technique and habit modification, such as discouraging lip biting, play a crucial role in long-term success.[15]

Alternative treatment modalities, including laser ablation, cryosurgery, and marsupialization, have been reported with

varying degrees of success. Laser excision offers advantages such as reduced intraoperative bleeding and postoperative discomfort, but conventional surgical excision remains a widely preferred approach due to its simplicity, low cost, and predictable outcomes. [7]

The present case series reaffirms the clinical characteristics, diagnostic process, and favorable prognosis of mucocoeles when managed appropriately. Early recognition, complete excision, and

addressing underlying etiological factors are key to preventing recurrence and ensuring optimal patient outcomes.

## CONCLUSION

Mucoceleles are common, benign lesions of the oral cavity that, while not life-threatening, can cause functional and aesthetic concerns for patients. The present case series highlights their typical occurrence on the lower lip, often associated with mechanical trauma or habitual lip biting, and emphasizes the importance of accurate clinical diagnosis confirmed by histopathological evaluation. Complete surgical excision, including removal of the associated minor salivary glands, proved to be an effective and reliable treatment modality, with no recurrences observed during follow-up. Early detection, elimination of predisposing factors, and meticulous surgical technique are essential to achieving optimal outcomes and preventing recurrence.

**Conflict of Interest:** There is no Conflict of Interest.

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## **Conservative Correction of Midline Diastema and Anterior Spacing with Lithium-Disilicate Veneers: A Case Report**

**Naeema Basar, Rahul Kulkarni, Vishwas Kharsan, Hema Padma Bala**

Department of Prosthodontics, Nair Hospital Dental College, Mumbai, Maharashtra – 400008, India.

Corresponding Author Email: [drnaemabasar@gmail.com](mailto:drnaemabasar@gmail.com)

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### **ABSTRACT:**

Adult patients frequently seek rapid esthetic correction of anterior spacing yet decline orthodontics. A 24-year-old male presented with midline diastema and generalized maxillary anterior spacing, Angle's Class III malocclusion, and healthy periodontium (Miller Class I recession). Facially driven planning with diagnostic models and mock-ups established proportional width-to-height targets for space redistribution from canine to canine. Enamel-oriented preparations with supragingival margins were completed, and lithium-disilicate veneers were CAD/CAM fabricated. Adhesive cementation followed hydrofluoric etching and silanization of the intaglio, selective enamel etching, and placement with a light-cure veneer resin to optimize color stability. The restorations achieved space closure, natural emergence profiles, and shade integration; occlusion was refined to stable anterior guidance. Short-term review showed healthy soft tissues with no debonding or marginal discoloration, and the patient reported high satisfaction. This case supports lithium-disilicate veneers as a predictable, minimally invasive alternative when orthodontic treatment is declined.

**KEY WORDS:** Lithium disilicate; Porcelain laminate veneers; Diastema closure; Minimally invasive dentistry

### **INTRODUCTION**

Anterior spacing, midline diastema, and mild proclination are frequent esthetic complaints that can also influence phonetics and anterior guidance.[1] Although orthodontic therapy is the most biologic means of correcting tooth position and space distribution, many adult patients decline comprehensive alignment because of time, cost, or personal preference.[2] Contemporary adhesive dentistry and high-strength glass-ceramics have therefore expanded conservative restorative pathways that can harmonize tooth proportions, close spaces, and improve smile esthetics while preserving the majority of enamel.[3]

Porcelain laminate veneers, particularly lithium disilicate, offer a favourable balance of optical realism and mechanical performance at minimal thicknesses.[4] When preparations remain largely in enamel and margins are kept supra- or equigingival, adhesive durability and periodontal compatibility

are enhanced.[5] Predictable bonding rests on meticulous protocols that include hydrofluoric acid conditioning of the ceramic, silanization, selective enamel etching, and the use of light-cure veneer cements to optimize color stability under thin restorations.[6]

Equally critical is facially driven planning. Diagnostic photographs, wax-ups/digital mock-ups, and proportion-based analyses (e.g., width-to-height ratios) guide tooth reduction, contact area placement, and emergence profiles to avoid overbulking during space redistribution. Digital workflows and CAD/CAM fabrication further assist in standardizing contours and interproximal relationships, improving try-in efficiency and shade control.[7]

The present case report outlines a minimally invasive rehabilitation of midline diastema and generalized maxillary anterior spacing in a young adult who declined orthodontics. It details the diagnostic rationale, enamel-oriented preparations, adhesive cementation of lithium-disilicate veneers, and short-

term outcomes, emphasizing the principles and clinical steps that support esthetic integration, functional harmony, and patient satisfaction.

**Case Presentation:** A 24-year-old male presented with the chief concern of an unesthetic smile due to spacing in the maxillary anterior region and requested correction. He reported no relevant medical history and had undergone routine oral prophylaxis six months earlier. General examination revealed an average build and a normal gait.

**Clinical findings:** Extraoral assessment showed a straight facial profile with an ovoid facial form and competent lips. Temporomandibular joint examination revealed no abnormalities. Intraorally, a midline diastema was present with generalized spacing from right canine to left canine in the maxillary arch (teeth 13–23). Bilateral Angle's Class III molar relation and canine relationships were recorded.

Localised gingival recession consistent with Miller's Class I was noted without signs of active inflammation. Smile analysis demonstrated that the lip line followed the smile arc, the face was grossly symmetric, and the patient displayed a medium smile line with a premolar-to-premolar natural smile. Micro-esthetic discrepancies were chiefly the midline diastema and the anterior interdental spacing.

**Diagnostic assessment:** A comprehensive clinical and radiographic evaluation was performed to exclude underlying pathology and confirm suitability for a conservative, enamel-preserving restorative approach aimed at space closure and smile enhancement. The esthetic analysis, occlusal scheme (bilateral Class I), and periodontal status (localized Miller Class I recession) supported a minimally invasive treatment.

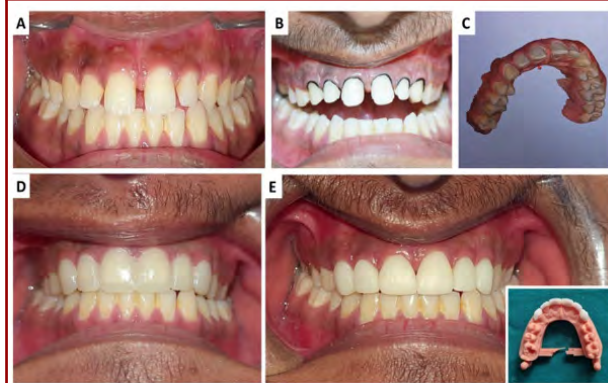
**Therapeutic options and shared decision-making:** Three options were discussed: (1) direct composite buildups, (2) porcelain laminate veneers (laminates), and (3) full-coverage restorations. The comparative advantages, anticipated longevity, tooth-structure preservation, costs, chairside time, reparability, and maintenance were explained. Given the patient's desire for improved longevity with minimal tooth preparation, lithium-disilicate laminate veneers involving the maxillary anterior teeth were elected. Written informed consent was obtained.

**Therapeutic intervention:** Shade selection was completed prior to tooth preparation using the VITA 3D-Master shade guide under natural light. Tooth preparation followed a minimally invasive protocol to maintain enamel as the primary bonding substrate. Provisionalization was achieved with a bis-acryl provisional material (OraTemp) to preserve esthetics and function during fabrication (Figure 1).

At the definitive appointment, provisionals were removed, the field was cleaned, and absolute isolation with a rubber dam was achieved. The definitive ceramic laminates were tried in and

evaluated for marginal fit, cervical extension, proximal contacts, and shade integration.

**Figure 1: A) Pre-operative Photograph; B) Gingival retraction after tooth preparation; C) Digital impression by intraoral scanning; D) Cementation of temporary crowns; E) Bisque trial (Inset: denture after bisque trial)**



The bonding protocol began with conditioning of the restorations: the intaglio surfaces were etched with 9% hydrofluoric acid for 90 seconds, thoroughly rinsed, and air-dried until a uniform frosty appearance was observed. Surface by-products were cleaned by applying 37% phosphoric acid for 60 seconds and rinsing, followed by application of a silane coupling agent for 60 seconds and gentle air-drying.

Tooth-side procedures involved selective enamel etching with 37% phosphoric acid for 20 seconds, thorough rinsing, and gentle air-drying to a frosty enamel surface. A universal adhesive was applied uniformly and gently air-thinned without light-curing to optimize interaction with the luting resin.

Each laminate veneer (13 to 23) was luted with a light-cure resin cement (3M™ RelyX™ Veneer Cement). The exclusive use of a light-activated cement was chosen to avoid color shifts associated with chemical initiators in dual-cure agents and because veneer thickness permits reliable photoactivation (Figure 2).

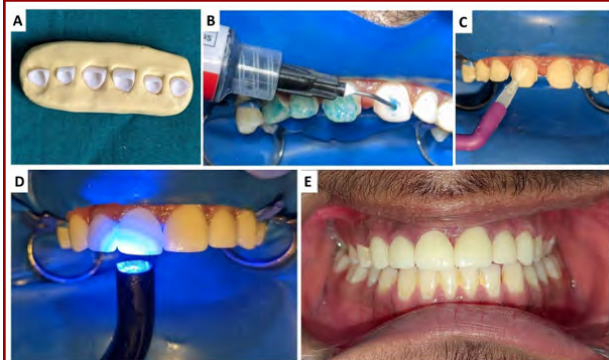
After careful seating under controlled pressure, excess cement was removed, and each restoration was light-cured from multiple aspects for adequate polymerization. Occlusion and excursions were verified and adjusted as needed, and margins were finished and polished.

**Follow-up and outcomes:** Immediate postoperative evaluation showed excellent marginal adaptation, appropriate cervical contours, harmonious shade matching, and closure of the diastema and interdental spaces with natural emergence and proportional tooth widths (Figure 3). Soft-tissue response at the margins was healthy with no bleeding on gentle probing.

The patient expressed high satisfaction with the esthetic outcome and smile harmony. Postoperative instructions regarding hygiene,

avoidance of parafunctional habits, and recall were provided, and the patient was scheduled for periodic reviews.

**Figure 2: A) Etched prosthesis; B) Etching of teeth; C) Application of bonding agent; D) Light curing after cementation of veneers; E) Final cemented prosthesis.**



**Figure 3: Comparative demonstration of A) Pre-operative Smile and B) Post-operative Smile**



**Patient perspective:** The patient reported feeling more confident with the appearance of his smile following closure of the anterior spacing and appreciated that the procedure preserved natural tooth structure while delivering a lifelike result.

## DISCUSSION

This case illustrates a conservative, patient-centred approach to midline diastema and generalized anterior spacing in a young adult who declined orthodontics. Lithium-disilicate laminate veneers were selected to redistribute spaces, correct tooth proportions, and harmonize the smile while preserving enamel and periodontal health.[8] Comprehensive planning, including diagnostic wax-up/digital mock-up and proportion-guided width-to-height adjustments, enabled controlled space closure without overbulking, maintained a natural smile arc, and respected lip support.[9]

Enamel-oriented preparations with supragingival margins, rounded internal angles, and calibrated incisal reduction maximized bond

predictability and soft-tissue compatibility, consistent with ultraconservative veneer concepts.[10]

Adhesive protocols were tailored to thin glass-ceramic restorations: hydrofluoric etching of the intaglio, phosphoric-acid cleansing, silanization, selective enamel etching, and rubber-dam isolation to create a stable micro- and chemical bond. [11] A purely light-cured veneer cement supported shade stability and facilitated excess removal through translucent ceramics, aligning with contemporary recommendations for laminate veneer cementation.[10]

Material selection balanced optics and strength; lithium disilicate provided lifelike translucency and sufficient flexural performance across multiple anterior units, an approach mirrored in recent esthetic-driven space-management reports that emphasize minimally invasive corrections when orthodontics is refused.[12] CAD/CAM workflow further standardized contours and proximal contacts during space redistribution and has been successfully used in comparable veneer cases to streamline delivery and esthetics.[13]

Functional finishing focused on establishing smooth anterior guidance and eliminating excursive interferences to reduce the risks of marginal chipping and incisal fractures. [14] Early outcomes, including excellent marginal adaptation, stable shade, healthy soft tissue, and high patient satisfaction, are consistent with published experiences when case selection, enamel-based preparation, and meticulous bonding are respected. [12] Limitations include technique sensitivity, susceptibility to parafunctional overload, and the need for patient adherence to maintenance.[15]

Nevertheless, for carefully selected patients prioritizing rapid esthetic improvement without orthodontics, lithium-disilicate laminate veneers offer a predictable, minimally invasive solution with favorable short-term performance and a biologically respectful profile. Periodic reviews remain essential to monitor occlusion, margins, and soft tissues and to reinforce protective behaviors.

## CONCLUSION

The present case report demonstrates that lithium-disilicate laminate veneers can conservatively correct midline diastema and generalized anterior spacing with excellent short-term esthetics, functional harmony, and soft-tissue health when guided by structured planning and enamel-based adhesive protocols. For patients who decline orthodontics, this minimally invasive approach is a viable alternative; however, long-term success remains contingent on careful case selection, occlusal management, and regular maintenance reviews.

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## Digital Custom Abutment for the Prosthetic Management of a Malpositioned Anterior Implant: A Case Report

**Dr. Rajeev Singh, Dr. Gaurang S Mistry, Dr. Rubina Tabassum,  
Dr. Sheetal B. Parab, Dr. Ashmita Chabria & Dr. Shresth Kapoor**

Department of Prosthodontics Institute of DY Patil University School  
of Dentistry, Nerul, Navi Mumbai, Maharashtra, India.

Corresponding Author Email: [drshresthkapoor24@gmail.com](mailto:drshresthkapoor24@gmail.com)

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### ABSTRACT:

Malpositioned dental implants in the anterior esthetic zone pose significant restorative and esthetic challenges. Conventional management often involves implant removal and re-placement with grafting, which can be invasive, time-consuming, and costly. This case report presents the prosthetic rehabilitation of a 26-year-old male with a malpositioned maxillary anterior implant managed using a digitally designed custom abutment. A digital impression was obtained and the abutment was designed using CAD software, followed by 3D printing of a resin prototype for trial verification. The definitive abutment was cast in cobalt-chromium alloy and restored with a porcelain-fused-to-metal crown incorporating gingival ceramic to compensate for soft tissue deficiencies. Esthetics were further enhanced with composite veneers on adjacent teeth. The prosthetic solution successfully corrected implant angulation, established a proper emergence profile, and provided natural esthetics and functional stability. This case highlights the clinical value of digital technologies and custom abutments in contemporary implant dentistry.

**KEY WORDS:** Custom abutment; malpositioned implant; CAD/CAM; digital dentistry; anterior esthetics.

### INTRODUCTION

Dental implant therapy has become a predictable treatment modality for replacing missing teeth, with success dependent not only on osseointegration but also on correct three-dimensional positioning of the implant fixture [1]. Proper implant placement is particularly critical in the anterior maxilla, where esthetic and functional demands are high [2]. Deviation from the ideal prosthetic axis in this region often results in restorative challenges, including compromised emergence profile, impaired soft tissue contours, and suboptimal esthetic integration with the adjacent dentition.

Malposition of implants may occur due to anatomical limitations, inadequate treatment planning, or intraoperative complications [3]. Traditional management of such cases

has involved implant removal followed by bone grafting and re-implantation in the correct position [4]. Although effective, these procedures are invasive, technique sensitive, time consuming, and associated with increased morbidity and patient cost [4,5]. An alternative prosthetic solution is the use of custom abutments, which allow correction of implant angulation, support of peri-implant tissues, and creation of a natural emergence profile without the need for additional surgery [6].

Recent advances in digital dentistry, including intraoral scanning, computer-aided design/computer-aided manufacturing (CAD/CAM), and additive manufacturing, have further enhanced the precision and predictability of custom abutment fabrication [7]. These technologies provide clinicians with the ability to design abutments that meet individualized esthetic and functional requirements in challenging scenarios [8]. The present case

report describes the prosthetic management of a malpositioned anterior maxillary implant using a digitally designed custom abutment, highlighting the clinical steps, material selection, and outcome.

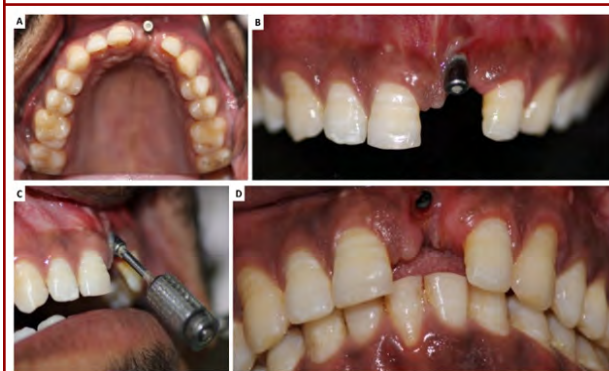
## Case Report

**Patient Information:** A 26-year-old male reported to the Department of Prosthodontics with a requirement of restoring a previously placed anterior maxillary implant. The patient was in good systemic health, with no significant medical history or contraindications to prosthodontic treatment. He expressed dissatisfaction with the esthetics of his smile and difficulty in obtaining a functional restoration due to the malpositioned implant.

**Clinical Findings:** Extraoral examination revealed a balanced facial profile with adequate lip support and no signs of temporomandibular dysfunction. Intraoral examination demonstrated a single implant fixture placed in the maxillary anterior region with a pronounced labial angulation. The implant platform was positioned approximately 3–4 mm labial to the ideal prosthetic position (Figure 1).

Despite the angulation, peri-implant soft tissues were healthy, and adequate keratinized tissue was present. Radiographic evaluation using periapical imaging confirmed satisfactory osseointegration without evidence of pathology, while also highlighting the extent of labial inclination.

**Figure 1: Pre-operative photograph A) Occlusal view and B) Frontal view; C) Checking Implant angulation; D) Sculpting of soft tissues around implant**



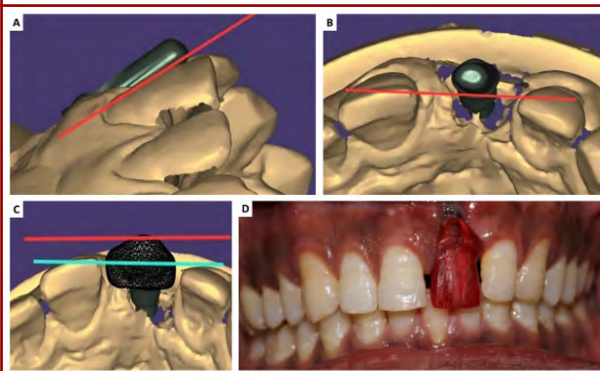
**Diagnostic Assessment:** Based on the clinical and radiographic findings, the case was diagnosed as a malpositioned anterior implant with labial angulation requiring prosthetic correction. Two options were considered: surgical removal with bone grafting and re-implantation, or a prosthetic approach using a custom angulated abutment. After discussing the risks and benefits of both, the patient consented to the prosthetic solution.

**Therapeutic Intervention:** The prosthetic rehabilitation began with digital impressions obtained using the open-tray

technique with polyvinyl siloxane impression material. The impressions were scanned to generate a digital model, which was used in CAD software to design a custom abutment capable of correcting the angulation and establishing an appropriate emergence profile.

A resin prototype of the abutment was fabricated through 3D printing and trial-fitted to verify adaptation, soft tissue response, and esthetics (Figure 2). After successful evaluation, the final abutment was cast in a cobalt-chromium alloy, finished, and polished for clinical use.

**Figure 2: A), B), and C) 3-D designing of the prosthesis; D) Trial of the 3D-Printed resin prosthesis**



Soft tissue sculpting was carefully performed during the trial and final placement phases to condition the peri-implant mucosa and optimize papillary form. The definitive prosthesis was fabricated as a porcelain-fused-to-metal crown incorporating gingival ceramic to compensate for soft tissue deficiency in the cervical region. The restoration was cemented with a resin-modified glass ionomer cement, ensuring proper retention and marginal seal. To further enhance esthetics and create harmony with the adjacent dentition, direct composite veneers were placed on teeth #11 and #22 using a nanohybrid composite system with enamel-bonding adhesive.

**Figure 3: A) Custom abutment and B) Cemented Final Prosthesis**



**Follow-Up and Outcomes:** The patient was reviewed at regular intervals to assess the health of peri-implant tissues, prosthesis integrity, and esthetic stability. The custom abutment corrected the unfavorable implant angulation and provided a natural emergence profile, while the PFM crown with gingival ceramic restored both esthetics and function (Figure 3). The adjacent composite veneers contributed to an improved smile line and overall harmony. The peri-implant tissues remained stable, with healthy mucosa and no signs of inflammation or bone loss. The patient expressed satisfaction with the functional and esthetic results.

## DISCUSSION

The management of malpositioned implants, particularly in the anterior maxilla, remains one of the most demanding challenges in implant prosthodontics due to the high esthetic expectations and the complex anatomical constraints of the region [4,9]. In the present case, the use of a custom abutment fabricated through a digital workflow allowed the correction of the labial angulation of the implant and provided an emergence profile that harmonized with the adjacent dentition.

This approach not only avoided the need for implant removal and grafting procedures, which are associated with increased morbidity and cost, but also preserved the osseointegration of the existing implant, thereby minimizing patient discomfort and treatment duration[10]. Custom abutments have been shown to provide distinct advantages over prefabricated stock abutments, especially when implant placement deviates from the ideal axis [8,11]. They facilitate precise correction of angulation, improved soft tissue support, and the development of natural gingival contours, which are critical to achieving esthetic success in the anterior zone [12].

The integration of CAD/CAM and 3D printing technologies further enhanced the predictability of the treatment by allowing virtual planning, prototyping, and clinical verification before final fabrication [13]. This digital workflow not only improved accuracy but also optimized communication between the clinician and the laboratory, leading to a more controlled and efficient process [13].

Material selection also played a significant role in the clinical success of the present case. The use of a cobalt-chromium alloy for the abutment provided adequate mechanical strength and biocompatibility, while the porcelain-fused-to-metal crown with gingival ceramic compensated for tissue deficiencies, ensuring seamless esthetics [14]. Additionally, the placement of composite veneers on adjacent teeth enhanced smile harmony, underscoring the value of a comprehensive esthetic approach [15].

The outcome of this case is consistent with existing literature that supports the use of custom abutments for malpositioned implants, with studies reporting high survival rates and long-term stability of peri-implant tissues when such prosthetic

solutions are employed. While the cost and technical demands of custom abutments may be higher compared to stock abutments, the benefits in terms of esthetics, function, and patient satisfaction justify their use in challenging cases [13-15]. Moreover, as digital technologies continue to evolve, the process of designing and fabricating custom abutments is becoming increasingly streamlined and accessible, further reinforcing their clinical relevance [10,11].

The present case, therefore, highlights the importance of careful treatment planning, patient-centered decision making, and the integration of modern digital tools in overcoming the limitations imposed by implant malposition. By adopting a prosthetic rather than surgical approach, predictable esthetic and functional outcomes were achieved while minimizing invasiveness, underscoring the value of custom abutment therapy in contemporary implant dentistry.

## CONCLUSION

The present case report demonstrates the successful rehabilitation of a malpositioned anterior implant using a digitally designed and custom-fabricated abutment, which corrected the implant angulation and restored a natural emergence profile. The prosthetic approach eliminated the need for invasive surgical intervention while achieving optimal esthetics, function, and patient satisfaction. Custom abutments, supported by digital technologies, represent a predictable and conservative solution for managing challenging implant positions in the esthetic zone.

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## **Comparative Evaluation of Abrasion Resistance of Two Type IV Die Materials with Three Different Die Hardening Agents: An *In vitro* Study**

**Dr. Hema Padma Bala<sup>1</sup>, Dr. Vishwas Kharsan<sup>2</sup>, Dr. Naeema Basar<sup>3</sup>,**

**Dr. Priyanka Chougule<sup>3</sup> & Dr. Rosy Shamkule<sup>3</sup>**

<sup>1</sup>Postgraduate Student, Department of Prosthodontics, Nair Hospital  
Dental College, Mumbai, Maharashtra, India

<sup>2</sup>HOD and Professor, Department of Prosthodontics, Nair Hospital  
Dental College, Mumbai, Maharashtra, India

<sup>3</sup>Department of Prosthodontics, Nair Hospital Dental  
College, Mumbai, Maharashtra, India

Corresponding Author Email: [hemabalach123@gmail.com](mailto:hemabalach123@gmail.com)

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### **ABSTRACT:**

Gypsum products are widely used as die materials in prosthodontics owing to their ease of use, accuracy, and cost-effectiveness. However, they are prone to surface abrasion, which may compromise the marginal integrity of restorations. Application of surface hardeners has been proposed to enhance abrasion resistance, though results in the literature remain inconsistent. To compare the abrasion resistance of two type IV die materials, Ultrabase and Pearlstone, with and without the application of three different die hardeners under controlled laboratory conditions. A total of 112 specimens were prepared using standardized carbon steel dies. The specimens were divided into eight groups (n=14 each): Ultrabase and Pearlstone uncoated (controls), and each material coated with Maarc, MDM, or 2GM die hardener. Abrasion resistance was tested using a custom-designed abrasion machine under loads of 20 g and 50 g.

Mean weight loss was calculated before and after abrasion. Data were statistically analyzed using ANOVA, Kruskal–Wallis, and independent t-tests, with significance set at  $p < 0.05$ . Uncoated specimens exhibited the highest mean weight loss, while all surface hardeners significantly reduced abrasion. At 20 g load, Pearlstone with Maarc coating showed the least weight loss. At 50 g load, Pearlstone coated with MDM demonstrated the greatest resistance. Overall, Pearlstone performed better than Ultrabase irrespective of the hardener used. Pooled analysis confirmed significantly lower mean weight loss in coated specimens compared to controls ( $p \leq 0.001$ ). Surface hardeners enhance the abrasion resistance of type IV gypsum dies, with Pearlstone showing superior performance over Ultrabase. Incorporation of surface coatings may improve the durability and accuracy of working dies in prosthodontic practice.

**KEY WORDS:** Die materials; Gypsum; Abrasion resistance; Surface hardeners

### **INTRODUCTION**

Gypsum products are among the most frequently employed materials in prosthodontics for the fabrication of definitive casts and dies [1]. Their popularity stems from their ease

of manipulation, cost-effectiveness, compatibility with various impression systems, and controlled setting expansion [1,2]. These characteristics make them highly suitable for producing accurate reproductions of oral structures and facilitating successful indirect restorative procedures [3].

Despite these advantages, gypsum products exhibit several inherent limitations, such as low fracture strength, dimensional instability, technique sensitivity, and most importantly, susceptibility to abrasion [4]. These shortcomings may compromise the marginal fidelity of restorations and ultimately affect their clinical longevity.

Surface abrasion is a significant concern in laboratory procedures. Repeated manipulations such as carving, finishing, or disinfection of gypsum dies can accelerate surface wear [5]. Marginal abrasion in particular may jeopardize the accuracy of fixed prostheses. Several approaches have been explored to improve the abrasion resistance of gypsum dies [6]. Epoxy resin has shown superior mechanical strength compared to gypsum-based materials; however, its use remains limited due to high cost and dimensional inaccuracy [7]. Other approaches, such as altering the mixing liquid with colloidal silica or soluble resin, have improved strength but introduced undesirable changes in setting expansion [8].

A more widely accepted method involves application of surface hardeners, including cyanoacrylate resins, colloidal silica, and commercial protective coatings. Fukui et al. demonstrated that cyanoacrylate coatings could improve surface hardness by forming a thin protective film, while Richardson highlighted the importance of maintaining optimal film thickness [9]. Lindquist et al. showed that abrasion resistance and water sorption were influenced by the specific combination of die material and hardener [10]. Similarly, Harris and associates observed that hardener coatings altered surface hardness, although results varied depending on the base material [11].

Given the growing availability of commercial surface hardeners and the inconsistencies reported in previous investigations, further comparative evaluation is necessary. The present in vitro study was designed to compare the abrasion resistance of two type IV die materials, UltraBase and Pearlstone, with and without the application of three different die hardening agents, in order to provide reliable evidence for clinical and laboratory practice.

## MATERIAL AND METHODS

**Study Design:** The present in vitro experimental study was conducted at the Department of Prosthodontics in collaboration with the Indian Institute of Technology, Bombay. The study protocol was approved by the institutional ethical review board (IEC-NHDC Project No:- EC-202/PROSTHO/ND13/2023, dated: 25/07/2023)

**Materials Used:** Two ADA type IV gypsum die materials were selected for the study: K Ultrabase and Pearlstone. Three die hardeners were chosen based on their clinical availability and widespread use: Maarc die hardener (Shiva Products Ltd., India), MDM die hardener, and 2GM die hardener. Uncoated specimens of both die stones served as control groups. All materials were procured from a single batch to eliminate batch-related variability.

**Preparation of Specimens:** A standardized carbon steel alloy die was fabricated with dimensions of  $50 \times 50 \times 8$  mm, containing 27 vertical ridges of 1 mm depth, each with an internal and external slope of  $45^\circ$  (Figure 1). These ridges were designed to simulate the margins of a prepared tooth (Figure 2). Impressions of the die were made using a putty-wash technique and evaluated for surface imperfections.

The die stones were mixed according to the manufacturer's instructions: distilled water was first placed in a vacuum mixing bowl, the powder added gradually, soaked for a few seconds, hand spatulated for 10 seconds, and subsequently mixed under vacuum for 20 seconds.

**Figure 1: Carbon steel alloy dies used to fabricate samples in the study**



**Figure 2: Ridges incorporated in the design of the dies**



The slurry was carefully incrementally poured into the impressions while placed on a mechanical vibrator set at 6000 cycles/min and 0.4 mm amplitude, to prevent entrapment of air bubbles. The filled metal moulds were placed on a glass slab to ensure a parallel base. After allowing the specimens to set for 1 hour at an ambient temperature of  $20 \pm 2^\circ\text{C}$  and relative humidity of  $50 \pm 10\%$ , the casts were retrieved. A total of 112 specimens were prepared, 14 for each subgroup (Figure 3). All specimens were stored at  $24\text{--}26^\circ\text{C}$  for 14 days to ensure complete drying.

**Grouping of Specimens:** The prepared specimens were divided into eight groups of 14 samples each:

- Group 1A: Ultrabase, uncoated (control)
- Group 1B: Pearlstone, uncoated (control)
- Group 2A: Ultrabase + Maarc die hardener

- Group 2B: Ultrabase + MDM die hardener
- Group 2C: Ultrabase + 2GM die hardener
- Group 3A: Pearlstone + Maarc die hardener
- Group 3B: Pearlstone + MDM die hardener
- Group 3C: Pearlstone + 2GM die hardener

**Figure 3: Prepared specimens**



Each group was further subdivided for abrasion testing under 20 g and 50 g loads.

**Application of Die Hardeners:** For the experimental groups, the respective die-hardener was applied uniformly to the surface of each specimen with a fine camel hair brush. Excess material was allowed to evaporate according to the manufacturer's guidelines. The control groups received no surface coating.

**Abrasion Resistance Testing:** Abrasion resistance was evaluated using a custom-designed abrasion testing machine based on the recommendations of Lindquist et al. The stylus of the machine was passed repeatedly across the ridges of each specimen under controlled load conditions. Weight loss of each specimen was recorded before and after abrasion using a precision electronic balance, and the difference was expressed in milligrams as an indicator of abrasion resistance.

**Statistical Analysis:** All collected data were entered into Microsoft Excel spreadsheets and analyzed using SPSS statistical software (version 23, IBM Corp., USA). Descriptive statistics, including mean and standard deviation, were computed for each group. Normality of data distribution was assessed using the Shapiro–Wilk test.

Depending on the distribution, comparisons among multiple groups were performed using one-way ANOVA with post-hoc Tukey's test for normally distributed data, or the Kruskal–Wallis test for non-parametric data. For paired group comparisons, the independent samples t-test or Mann–Whitney U test was employed as appropriate. A p-value of <0.05 was considered statistically significant.

## RESULTS

**Descriptive Statistics under 20 g Load:** A total of 112 specimens were evaluated for abrasion resistance, divided equally between two die materials (Ultrabase and Pearlstone) with and without

the application of three different die hardeners. The initial analysis focused on weight loss recorded under a 20 g load. The mean weight loss values for each group are presented in Table 1. Uncoated specimens demonstrated the highest weight loss in both die materials, indicating greater susceptibility to abrasion.

**Table 1. Mean weight loss (mg) of specimens under 20 g load**

Die Material	Uncoated	Maarc Hardener	MDM Hardener	2GM Hardener
Ultrabase	2.24	1.36	1.41	1.26
Pearlstone	2.01	0.79	0.99	1.19

In contrast, application of die hardeners consistently reduced the mean weight loss. Among the Ultrabase groups, the lowest value was observed with 2GM die hardener (1.26 mg), followed closely by Maarc (1.36 mg) and MDM (1.41 mg). For Pearlstone, the lowest mean weight loss was seen with Maarc (0.79 mg), while MDM and 2GM also demonstrated reduced values compared with the uncoated control. Overall, Pearlstone specimens exhibited lower mean weight loss compared to Ultrabase across all groups. The greatest reduction in abrasion was noted in Pearlstone coated with Maarc hardener, suggesting superior performance of this combination under a lighter load.

**Descriptive Statistics under 50 g Load:** When specimens were subjected to a 50 g load, all groups showed increased weight loss compared to their counterparts tested at 20 g. The mean values are summarized in Table 2. For Ultrabase specimens, the uncoated control exhibited the highest mean weight loss (2.94 mg).

Application of die hardeners significantly reduced weight loss, with Maarc (1.60 mg) and 2GM (1.64 mg) demonstrating better performance than MDM (1.86 mg). Similarly, Pearlstone specimens showed a substantial reduction in abrasion with surface hardeners compared to the uncoated control (2.98 mg). Among these, MDM produced the lowest mean weight loss (0.90 mg), followed closely by Maarc (0.96 mg), while 2GM showed slightly higher values (1.66 mg).

**Table 2. Mean weight loss (mg) of specimens under 50 g load**

Die Material	Uncoated	Maarc Hardener	MDM Hardener	2GM Hardener
Ultrabase	2.94	1.60	1.86	1.64
Pearlstone	2.98	0.96	0.90	1.66

On inter-material comparison, Pearlstone once again demonstrated lower weight loss values than Ultrabase when coated with hardeners, particularly in the MDM and Maarc groups. The

findings reaffirm the protective effect of surface hardeners under higher load conditions, with Pearlstone showing a more favorable response compared to Ultrabase.

**Comparative Statistical Analysis:** Statistical testing revealed that within the 20 g load groups, the reduction in weight loss was significant for MDM-coated Ultrabase specimens compared to the uncoated control, whereas no significant difference was detected in the 2GM and Maarc subgroups. For Pearlstone, the application of Maarc hardener produced the most significant reduction in abrasion values.

At the 50 g load, significant improvement in abrasion resistance was observed in 2GM-coated specimens compared to controls, while differences among the other hardeners varied depending on the die material tested. Inter-material comparisons indicated that Pearlstone consistently exhibited lower weight loss values than Ultrabase irrespective of the type of surface treatment, and this finding was statistically significant ( $p = 0.006$ ).

When pooled data of all coated groups were compared against uncoated controls, specimens treated with die hardeners demonstrated markedly lower mean weight loss. This difference was found to be highly significant ( $p \leq 0.001$ ), confirming the overall protective effect of surface treatments on the abrasion resistance of gypsum dies.

## DISCUSSION

The present study evaluated the effect of three commercially available surface hardeners on the abrasion resistance of two type IV die materials, Ultrabase and Pearlstone. Abrasion resistance was measured under two different loads, 20 g and 50 g, to simulate varying laboratory conditions that may occur during finishing, adjustment, and routine handling of dies. The findings revealed that application of surface hardeners consistently improved the abrasion resistance of both die materials, with Pearlstone exhibiting superior performance compared to Ultrabase across most experimental conditions.

The reduction in weight loss among coated specimens can be attributed to the formation of a protective surface layer that resists wear under mechanical stress [12,13]. Hardening agents such as cyanoacrylates and resin-based coatings are known to penetrate the superficial microporosities of gypsum and polymerize, forming a barrier that distributes load and minimizes fracture propagation [9,14,15].

In the present study, the differences between the three hardeners suggest that the chemical composition and penetration ability of each material play a critical role in determining effectiveness. For example, Pearlstone treated with Maarc and MDM demonstrated the least abrasion under higher loads, likely due to more uniform film formation and deeper surface penetration compared to 2GM.

These observations are consistent with earlier reports. Lyon et al. (1987) demonstrated significant differences in abrasion resistance

among various die stones, emphasizing the influence of material composition [16]. Lindquist et al. (2002) found that resin-impregnated stones did not significantly differ from conventional stones in abrasion resistance but showed reduced material loss, suggesting a protective effect similar to that observed with surface coatings [17]. In a subsequent study, Lindquist et al. (2003) reported that improvements in abrasion resistance and reductions in water sorption occurred only with specific material–hardener combinations, corroborating the current findings that not all surface treatments are universally effective [10].

Harris et al. (2004) noted that die hardener coatings could paradoxically reduce surface hardness in certain gypsum products, highlighting the variability in outcomes depending on the interaction between base material and coating [11]. In contrast, He et al. (2010) demonstrated that while penetration of hardeners did not substantially alter intrinsic mechanical properties, the presence of a surface film effectively protected the die surface from abrasion damage [18]. These findings align with the present study, where the protective layer provided by hardeners significantly reduced mean weight loss compared to uncoated controls [19].

Pearlstone consistently outperformed Ultrabase in terms of abrasion resistance across different hardener groups. This may be related to its finer particle size distribution and denser microstructure, which provide a stronger base for surface coating [10,20]. Similar results were reported by Singh et al. (2018), who observed that abrasion resistance varied among different brands of type IV die materials and that certain materials exhibited superior performance when combined with specific surface hardeners [21]. The clinical implications of this study are noteworthy.

Marginal accuracy of fixed prostheses depends heavily on the integrity of the working dies [22]. Surface abrasion during laboratory procedures can lead to marginal discrepancies that compromise restoration fit [23,24]. The use of surface hardeners therefore represents a practical and cost-effective method to improve the durability of dies and ensure better clinical outcomes [25]. The results of this study reinforce the recommendation that application of surface coatings should be considered routine practice in prosthodontic laboratories, especially when using type IV die stones for fabricating precision restorations.

Despite its valuable findings, this study has certain limitations. It was conducted under *in vitro* conditions, which may not entirely replicate the complex variables encountered in clinical and laboratory settings. Only two brands of type IV die materials and three surface hardeners were tested, which may not represent the full range of products available commercially.

In addition, only short-term evaluation of abrasion resistance was performed, without assessing the long-term stability of the coatings under repeated cycles of use and disinfection. Future studies incorporating a larger variety of materials, long-term aging protocols, and different modes of mechanical testing would provide more comprehensive insights.

## CONCLUSION

Within the limitations of this in vitro study, it can be concluded that the application of surface hardeners significantly improves the abrasion resistance of type IV die materials. Among the tested combinations, Pearlstone coated with Maarc and MDM die hardeners demonstrated the least weight loss, while Ultrabase specimens also benefited from surface treatment though to a lesser degree. Overall, Pearlstone exhibited superior resistance compared to Ultrabase, irrespective of the hardener used. These findings highlight the clinical relevance of employing surface hardeners to enhance the durability and accuracy of working dies, thereby improving the reliability of prosthodontic procedures.

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## **Meta-Analysis of the CTLA-4 A/G Genetic Polymorphism Association and Risk of Graves' Disease**

**Veeramuthumari P**

Corresponding Author details: Assistant Professor of Zoology, V.V.Vanniaperumal

College for Women, Virudhunagar, Tamil Nadu India.

Corresponding Author Email: [muthusdream@gmail.com](mailto:muthusdream@gmail.com)

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### **ABSTRACT:**

Human CTLA-4 gene located on 2q33 encodes a molecule which plays an important role in the down regulation of CD28 interaction with the ligands on the surface of antigen-presenting cells (APCs). Many studies showed the association between the CTLA4 exon-1 49A/G polymorphism and the risk of developing Graves' disease. In recent years many new studies were published which helped to shed light on the relationship of CTLA4 SNP49 with GD. So the present study performed the meta-analysis to explore the association between the SNP49 and GD susceptibility in human beings. At presently there are several SNP public databases and SNP is found to be widely used in the genetic association studies of various complex diseases such as obesity, diabetes, osteoporosis, asthma, hypertension, kidney failure and thyroidism etc. The insilico study found that the CTLA4 49A/G SNP in exon-1 leads to the substitution of Ala with Thr in the signal peptide part.

CTLA-4 is the one of widely studied non-HLA susceptibility gene of GD, is mainly expressed on the surface of Treg cells and conventional T cells and suppresses self-reactive T cell responses via down regulating ligand availability for the co-stimulatory receptor CD28 to elicit inhibitory signals. The current study concluded that the polymorphisms of CTLA-4 are to be the candidates of the risk of the common autoimmune diseases at the genetic level. As GD is a T cell mediated autoimmune disorder and CTLA-4 plays a vital role in regulating T cell function. The study suggested that CTLA-4 expression or function is most likely associated with the pathogenesis of GD. Single nucleotide polymorphisms in the CTLA-4 gene may contribute to abnormal levels of CTLA-4, and subsequently play a leading part in the susceptibility to GD and still more SNPs research could be implemented for the betterment of the human health in future.

**KEY WORDS:** Allele, Single Nucleotide Polymorphism, Autoimmune Disease, Thyroidism and Gene.

### **INTRODUCTION**

Thyroid disorders are appearing to be the leading major endocrine disorders. Thyroid dysfunction affects a significant portion of the population and appears to be the leading endocrine disorder. In India, thyroid diseases are common worldwide. [5, 7]. According to a projection from

various studies on thyroid disease, it has been estimated that about 42 million people in India suffer from thyroid diseases. The literature data reported that on the epidemiology of five common thyroid diseases in India: hypothyroidism, hyperthyroidism, goiter and iodine deficiency disorders, hashimoto's thyroiditis, and thyroid cancer. Thyroid disorders are affecting around 42 million people in India [6, 13].

The thyroid is composed of closed sacs (follicles) lined with specialized thyroid cells. These cells secrete thyroglobulin, a large protein that acts as a storage molecule from which thyroid hormones are made and released into the blood. The rate at which this occurs is regulated by thyroid-stimulating hormone (TSH), which activates the thyroid cells by combining with TSH receptors found on the thyroid cell membrane. Hashimoto disease involves swelling of the gland (a condition called goiter) and a loss of thyroid hormone production (hypothyroidism). The autoimmune process underlying this disorder is thought to be instigated by helper T cells that react with thyroid antigens, although the mechanism is not completely understood. Once activated, the self-reactive T cells stimulate B cells to secrete antibodies against several target antigens, including thyroglobulin [9].

Autoimmune thyroid diseases (AITD) are the most prevalent organ-specific autoimmune diseases (ADs) and affect 2 - 5% of the population with great variability between genders (i.e., women 5–15% and men 1–5%) [4]. AITD include Graves' disease (GD) and Hashimoto Thyroiditis (HT), among others. HT and GD are the major causes of hypothyroidism and hyperthyroidism, respectively [12]. Graves' disease (GD) is an autoimmune thyroid disease. Multiple genetic factors are believed to be involved in its pathogenesis, but the factors are largely unknown, except for sex (female disease preponderance) and the role of human leukocyte antigen (HLA) genes on chromosome 6.

To understand the mechanisms underlying the development of GD, a search for non-HLA-linked genes is crucial, and we tested several candidate genes, including the CTLA-4 gene on chromosome 2q33. CTLA-4 molecules may either facilitate or down-regulate the second signal to T-cells, which is provided by the interaction between the two accessory molecules CD28 and B7 [14]. So, the CTLA-4 is one of the candidates as genetic markers for autoimmune diseases and it has been reported that if it is any type of single nucleotide (SNPs) polymorphisms occurred in CTLA4 molecule found to be block the B7-CD28 interaction by acting as functional receptor for B7 antigens and thus can interfere with the co-stimulation signal and inhibit T-cell proliferation.

The most frequent type of human genome variation are single nucleotide polymorphisms (SNPs) providing powerful tools for a variety of medical genetic studies CTLA-4 acts by delivering an inhibitory signal decreasing cytokine production, activation and proliferation of T lymphocytes. Single nucleotide polymorphisms, frequently called SNPs are the most common type of genetic variation among people. Each SNP represents a difference in a single DNA building block, called a nucleotide. For example, a SNP may replace the nucleotide cytosine (C) with the nucleotide thymine (T) in a certain stretch of DNA. SNPs occur normally throughout a person's DNA. They occur almost once in every 1,000 nucleotides on average, which means there are roughly 4 to 5 million SNPs in a person's genome.

The Scientists have found more than 600 million SNPs in populations around the world. SNPs are found in the DNA between genes and it could be act as biological markers, helping scientists locate genes that are associated with disease. When SNPs occur within a gene or in a regulatory region near a gene, they may play a more direct role in disease by affecting the gene's function (<https://medlineplus.gov/genetics/understanding/genomicresearch/snp/>).

SNPs are helped to predict an individual's response to certain drugs, susceptibility to environmental factors such as toxins, and risk of developing diseases. It also used to track the inheritance of disease-associated genetic variants within families. Research is ongoing to identify SNPs associated with complex diseases such as heart disease, diabetes, and cancer. Single nucleotide polymorphisms (SNPs) are the most common source of genetic variation in eukaryotic species and have become an important marker for genetic studies.

Still now there is a need more research on SNPs analysis (Insilico) in human genes in India. Hence the study focused on the Single nucleotide gene polymorphism in CTLA-4 gene and A/G polymorphism at position 49. Hence the study aimed to identify the Single nucleotide polymorphisms and analyze the effect of SNPs in CTLA-4 gene function of Graves' disease condition in human.

## METHODOLOGY

The human CTLA-4 gene A/G polymorphism at position 49 in exon 1 and wild sequences were analyzed and confirmed with SNP data base, Online Mendelian Inheritance in Man (OMIM) available at National Centre for Biological Information (NCBI) website. The sequences used in this study were retrieved from the databases at the NCBI, EBI and SNP.

**Entrez and gene Ensembl:** These two are comprehensive sites, which organized, collective resource linking out to various tools providing general information of gene structure, expression, splice variants encoded proteins, regulatory elements and single nucleotide polymorphisms (SNPs). OMIM: Online Mendelian Inheritance in Man database useful to establish or investigate disease association of gene of interest [1].

**Phylogenetic Tree:** <https://www.ebi.ac.uk/Tools/services/rest/clustalo/result/clustalo-E20230416-072829-0392-86801791-p1m/phyloree>. Percent Identity Matrix: <https://www.ebi.ac.uk/Tools/services/rest/clustalo/result/clustalo-E20230416-072829-0392-86801791-p1m/pim>.

**Mview Visualization file:** <https://www.ebi.ac.uk/Tools/services/rest/mview/result/mview-E20230416-073924-0633-13120070-p1m/aln.html>. CTLA-4 (Benchling Result): <https://benchling.com/s/seq-cxPSfTOIgFuuygiUIs7c?m=slm-PMINsZn26fj4YXDtbBZd>. CTLA-4 Manipulated – Single nucleotide change in 49 th position of Exon 1 (Benchling Result): <https://benchling.com/s/seq-zoGIRFuooVtuQVh9OJ3Q?m=slm-zgs96S08DtElSKZry1Ug>

**Table 1. List of databases and Internet links:**

Databases	Link	Comments
GenBank	<a href="http://www.ncbi.nlm.nih.gov/GenBank/index.html">http://www.ncbi.nlm.nih.gov/GenBank/index.html</a>	Genetic sequence database, a collection of all publicly available DNA sequences
BLAST	<a href="http://www.ncbi.nlm.nih.gov/BLAST/">http://www.ncbi.nlm.nih.gov/BLAST/</a> (updated2014)	Finds regions of local similarity between sequences.
CLUSTALW	<a href="http://www.ebi.ac.uk/clustalw/">http://www.ebi.ac.uk/clustalw/</a> (updated 2014)	Multiple sequence alignment
Domain search	<a href="https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi">https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi</a>	Protein family and domains
SABLE	<a href="http://sable.cchmc.org/">http://sable.cchmc.org/</a> .	Solvent accessibility
SNP database	<a href="http://www.ncbi.nlm.nih.gov/snp">http://www.ncbi.nlm.nih.gov/snp</a>	Identify the SNPs in various regions of the genes.
Uniprot	<a href="http://www.uniprot.org">http://www.uniprot.org</a>	provides a database for protein information

## RESULTS AND DISCUSSION

Graves' disease (GD) is also known as toxic diffuse goiter which increases the level of thyroid hormone. It is one of the organ-specific autoimmune diseases and it accounts for 85% of all clinical hyperthyroidism. The study found that the disease often presents

in patients aged from 20–40 years old and with a male to female ratio of approximately 1:8 and a significant familial tendency. The clinical performance of GD is not limited to the thyroid but is a multi-system syndrome, including the high metabolic syndrome group, diffuses goiter, eye symptoms, lesions and thyroid extremity diseases.

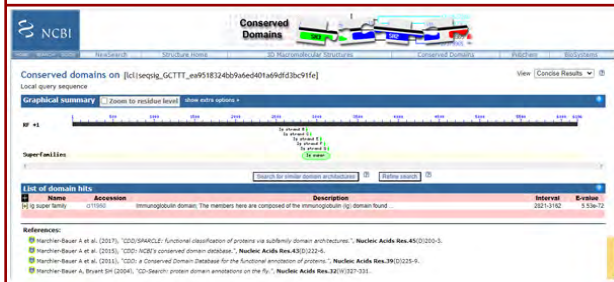
**Table 2. A/G SNPs in CTLA-4 gene**

Gene	Domain	Mutation	Amino acid position	Wild type	Mutant	Allelic frequency
CTLA-4	Ig super family	Missense	49	A	G	0.23

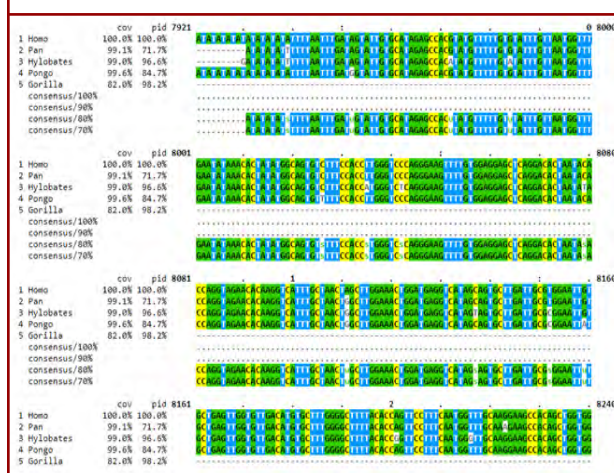
**Table 3. Uniprot and SNP web database of CTLA-4 gene:**

Assign Number	Alleles	Chromosome	Canonical Spdi	Gene	Functional Consequence	Clinical Significance	Validated	Maf
rs231775 [Homo sapiens]	A>G,T	2:203867991 (GRCh38) 2:204732714 (GRCh37)	NC_000002.12:203867990:A:G;NC_000002.12:203867990:A:T	CTLA4	missense_variant,coding_sequence_variant	benign,risk-factor	by frequency,by alfa,by cluster	G=0.371744/109761 (ALFA) G=0.208333/45 (Qatari) A=0.23913/11 (Siberian)
rs199912925 [Homo sapiens]	A>G	2:203871485 (GRCh38) 2:204736208 (GRCh37)	NC_000002.12:203871484:A:G	CTLA4	intron_variant,coding_sequence_variant,missense_variant	uncertain-significance	by frequency,by alfa,by cluster	G=0.000025/4 (ALFA) G=0.000013/1 (PAGE_STUDY) G=0.000033/4 (ExAC)

**Figure 1: Domain search**



**Figure 2: Multiple Sequence Alignment**

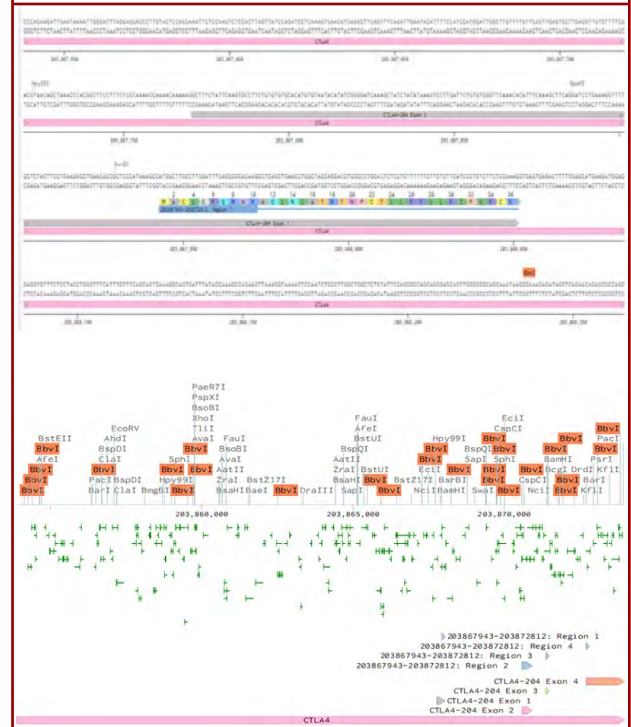


**Table 4. CTLA-4 gene sequence based prediction tools:**

Web Server	Effect
SNP database	Missense
Variant effect predictor	CTLA-4 gne
Uniprot/Swiss-prot	Single nucleotide polymorphism
(DM)2 – Domain Mapping & Disease mutation	Polymorphism
SIFT	Tolerated (Score: 0.08)
PolyPhen	Benign (Score:0.006)
PolyDoms	Nonsynonymous & Synonymous
MuSTAB	Disease

The study also demonstrated that the immunological concepts, GD are characterized by increased circulating antibodies against thyroid-stimulating hormone receptor (TSHR), thyroglobulin (TG) and thyroid peroxidase (TPO). Although the precise pathogenesis involved in the process of GD is not completely understood, certain findings indicate that complex interactions between environmental, genetic, endogenous and local factors are involved in its pathogenesis. Hence both immune-modulating genes and thyroid-specific genes are involved in its genetic pathogenesis.

**Figure 3: Visualization of Normal CTLA-4 (Benchling Result)**



It also remains unclear, however, how the interactions of various susceptibility genes contribute to the pathogenesis and clinical severity of the disease. So, the purpose of this study was to investigate the relationships between GD and A/G single nucleotide polymorphisms (SNPs) from CTLA-4.

Single Nucleotide Polymorphism (SNP) number search: CTLA-4 gene, function, accession number, name, protein name, number, taxonomic identifier number, taxonomic lineage, the total number amino acid, sequence status, disease, binary interaction, variant number, domain were found in Uniprot and SNP web database and it is shown in the table (Table: 2). The single nucleotide polymorphisms in CTLA-4 gene are important role in cause of the Graves' disease. The present study demonstrated a single nucleotide polymorphism (SNP) in CTLA 4 gene at position (amino acid) 49, the amino acid change is alanine (A) to threonine (G) nucleotide change. Both these (SNPs) are the missense mutation noted (Table:2 & 3). The study found that the occurrence of A/G polymorphism is high (57%) when compared to the C/T (43%) polymorphism in CTLA- 4 gene (Figure 1).

The multiple sequence alignment of CTLA-4 gene was carried out by CLASTALW. The evolutionary conservation at the position 49 (A/G) in which the missense mutation was observed revealed that this particular polymorphism is conserved across the species (Homo sapiens, Pongo abelii, Pan troglodytes, Gorilla gorilla gorilla, Hylobates moloch) (Figure:2).

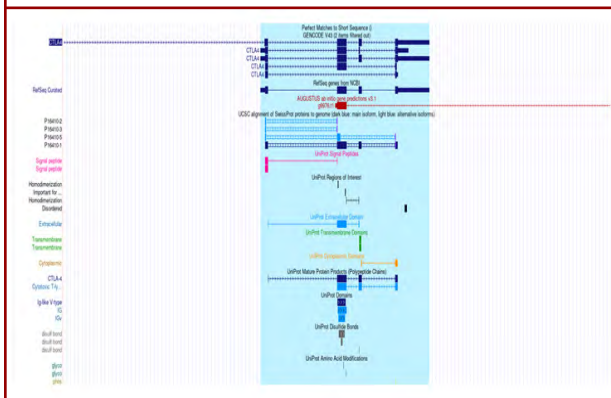
**Note:** Highlighted with yellow colour is found to be conserved across the species.

Benchling Webserver (For primary analysis) showed that Benchling digitizes data capture, workflows, and handoffs so scientists can simplify, standardize, and seamlessly share data easily across an organization. Bioedit (Sequence Manipulation) is a software program that embeds the tools that scientists and technicians need so they perform specific tasks, such as manipulation of sequence alignment, ABI tracing or RNA analysis. UCSC Genome Browser UCSC Genome Browser Interactively visualizes genomic data. Ensembl is a genome browser for vertebrate genomes that supports research in comparative genomics, evolution, sequence variation and transcriptional regulation. Ensembl annotate genes, computes multiple alignments, predicts regulatory function and collects disease data. Ensembl tools include BLAST, BLAT, BioMart and the Variant Effect Predictor (VEP) for all supported species (Table:4; Figure 3,4,5 & 6) .

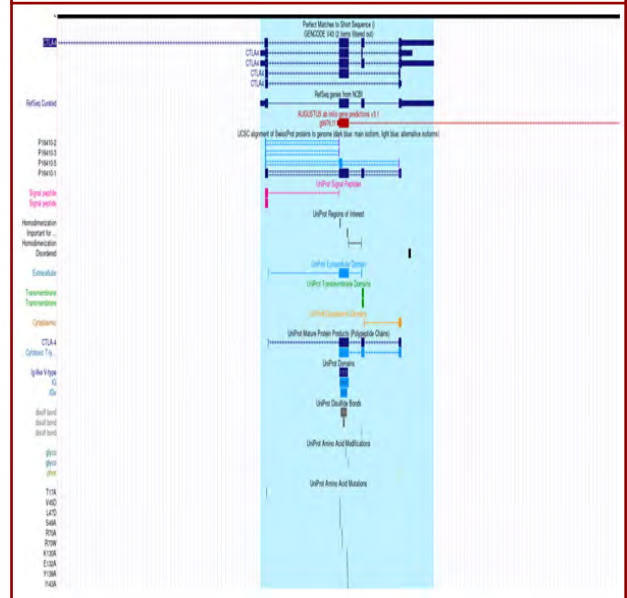
**Figure 4: Visualization of Manipulated CTLA-4 Gene (Benchling Result)**



**Figure 5 Visualization of Normal CTLA-4 (UCSC Result)**



**Figure 6: Visualization of Manipulated (UCSC Result)**



While visualizing, select the region of interest and use zoom into option for better understanding of the structure, restriction sites, ORF, Cut sites and Inline labels. For the confirmation the normal and changed *ctla-4* sequence structure was visualized in NCBI Genome Browser, ensembl and the resulting structure was as same as the Benchling result. Eventhough single nucleotide mutation in the *ctla-4* gene (Especially in 49 th position in Exon 1) showed that positive affection to Grave's disease, there is now considerable structural difference other than the changes in Aminoacid. One of the reasons for this may be the presence of CDS region in the terminal part of Exon 1, other than being in the 49 th position or nearby position. The meta analysis of CTLA-4 gene sequence compared with the manipulated (Single nucleotide A/G polymorphic sequence) showed that the changes in the sequence may alter the structure of the gene. It will lead to malfunctioning of the CTLA-4 gene during the immunological process.

Apart from GD, the polymorphism of CTLA-4 has been found in many diseases which included Addison's disease, autoimmune hypothyroidism, and rheumatoid arthritis. The CTLA4 49A/G SNP in exon-1 leads to the substitution of Ala with Thr in the signal peptide part which was reported to cause misprocessing of CTLA-4 in the ER resulting in less efficient glycosylation and diminished surface expression of CTLA-4 protein. According to [2,10] findings, the current study also demonstrated that the longer repeats of the UTR microsatellite are associated with reduced CTLA-4 inhibitory function.

Zhang et al., [15] reported that a number of studies have revealed that an autoimmune response, in particular cellular immunity against T lymphocytes and tyrosinase family proteins, acts a pivotal part in the initiation and maintenance of uveitis [11]. Lymphoid tyrosine phosphatase (LYP) is a member of the protein tyrosine kinases family and expressed in T cells The LYP protein is encoded by the human protein tyrosine phosphatase nonreceptor

22 (PTPN22) gene on chromosome 1p13, which plays a negative regulatory role in the T-cell signaling pathway. Dysfunction of PTPN22 (i.e., increased activity) suppressed the T-cell receptor (TCR) signal transduction of regulatory T cells, thus weakening the regulation function of T cells and leading to autoimmune diseases.

Many functional mutations have been described and conferred either altered susceptibility to autoimmune diseases or appeared to influence the severity and clinical outcomes Rawlings et al., 2015). Based on the literatures there is need of more research on association of SNPs and CTLA-4 molecule and related proteins. So, the study will concentrate on the proteins involved signal transduction of regulatory T cells in future.

## CONCLUSION

Human CTLA-4 gene, located on 2q33, encodes a molecule which plays an important role in the down regulation of CD28 interaction with the ligands on the surface of antigen-presenting cells (APCs) (8). The important inhibitory role of CTLA-4 in T-cell function has made it be one candidate gene when exploring autoimmune diseases. Many studies have established that T-lymphocyte antigen-4 (CTLA4) is a susceptible gene for Graves' disease (GD). Also many studies showed the association between the CTLA4 exon-1 49A/G polymorphism and the risk of developing Graves' disease. But those results were inconsistent. In recent years many new studies were published which helped to shed light on the relationship of CTLA4 SNP49 with GD.

So the present study performed the meta-analysis to explore the association between the SNP49 and GD susceptibility in human beings. At presently there are several SNP public databases and SNP is found to be widely used in the genetic association studies of various complex diseases such as obesity, diabetes, osteoporosis, asthma, hypertension, kidney failure and thyroidism etc. The allele frequencies are important in selection of SNPs for studying complex diseases [3]. The insilico study found that the CTLA4 49A/G SNP in exon-1 leads to the substitution of Ala with Thr in the signal peptide part. Cytotoxic T lymphocyte-associated protein 4 (CTLA-4), one of widely studied non-HLA susceptibility gene of GD, is mainly expressed on the surface of Treg cells and conventional T cells and suppresses self-reactive T cell responses via down regulating ligand availability for the co-stimulatory receptor CD28 to elicit inhibitory signals.

The current study concluded that the polymorphisms of CTLA-4 are to be the candidates of the risk of the common autoimmune diseases at the genetic level. As GD is a T cell mediated autoimmune disorder and CTLA-4 plays a vital role in regulating T cell function. The study suggested that CTLA-4 expression or function is most likely associated with the pathogenesis of GD. Single nucleotide polymorphisms in the CTLA-4 gene may contribute to abnormal levels of CTLA-4, and subsequently play a leading part in the susceptibility to GD and still more SNPs research could be implemented for the betterment of the human health in future.

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**Conflict of Interest:** Identification of genetic markers for the human diseases helped for the better diagnosis and treatment of the diseases.

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Associate Prof. Dr. Hülya CELİK Phd  
Agri Ibrahim Cecen University  
Faculty of Pharmacy / Department of Basic  
Pharmaceutical Sciences / Division of Basic  
Pharmacy Sciences 04100 Ağrı, Türkiye  
E-mail: [hycelik@agri.edu.tr](mailto:hycelik@agri.edu.tr)

### **MANAGING EDITOR**

**Dr. Gulafsha Kassab PhD (Biotechnology)**  
Department of Biotechnology, Saifia Science,  
College, Barkatullah University Bhopal India  
Email: [gulafshakhan.199410@gmail.com](mailto:gulafshakhan.199410@gmail.com)

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### **Mrs Nisha**

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