Author's response to reviews

Title: Circulating Immune Complexes and trace elements (Copper, Iron and Selenium) as markers in oral precancer and cancer: A randomised controlled clinical trial

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TITLE

Circulating Immune Complexes and trace elements (Copper, Iron and Selenium) as markers in oral precancer and cancer:

A randomized controlled clinical trial.

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KEY WORDS: Immune complexes, copper, iron, selenium, precancerous condition, India.
ABSTRACT

AIM: To evaluate the levels of circulating immune complexes, trace elements (copper, iron and selenium) in serum of patients with oral submucous fibrosis (OSMF), oral leukoplakia (L), and oral squamous cell carcinoma (OSCC), analyze the alteration and identify the best predictors amongst these parameters for disease occurrence and progression.

Methods: Thirty patients with oral submucous fibrosis / oral leukoplakia and 30 patients with oral squamous cell carcinoma, with histopathologically proven lesions were included in this study. For comparison thirty normal subjects were also selected. Proforma for the study was prepared and informed consent was taken from the subjects and controls. Clearance was obtained from the B.Y.L. Charitable Nair Hospital Ethical Committee (Nair hospital golden jubilee research foundation). Patients were selected randomly as per the inclusion criteria. The age group of these patients ranged from 25-70 years. Circulating immune complexes (CIC) were estimated using 37.5% Polyethylene Glycol 6000(PEG) serum precipitation. Serum estimation of copper (Cu), Iron (Fe) and selenium (Se) was done using the Oxalyl Dihydrazide method, Colorimetric Dipyridyl method and the Differential Pulse Cathodic Stripping Voltametry respectively.

Statistical Methods: The data was subjected to statistical analysis using the Chi Square Test, Standard Deviation, Student’s unpaired t-test, correlation, ANOVA and Linear Regression.

Results: The data analysis revealed increased circulating immune complex levels in the precancer and cancer patients. Serum copper levels showed
gradual increase from precancer to cancer patients. However, serum iron levels were decreased significantly in the cancer group. Selenium levels showed marked decrease in the cancer group. Among CIC, serum, copper, iron and selenium the best predictors for the occurrence of lesions were age, serum iron, CIC, serum selenium in the decreasing order.

**Conclusion:** The present study shows that these immunological and biological markers may be associated with the pathogenesis of oral premalignant and malignant lesions and their progressions. Concerted efforts would, therefore, help in early detection, management, and monitoring the efficacy of treatment.

Oral cancer the sixth most common cancer worldwide continues to be most prevalent cancer related to the consumption of tobacco, alcohol and other carcinogenic products.\(^1\) While the cancer remains high in South and South East Asia (its traditional high rise areas); parts of Central and Eastern Europe are seeing alarming increase and now constitute the highest incidence parts of the globe.\(^2\)

Increasing awareness on part of the providers of treatment, as well as the population in general, has led to a large proportion of patients presenting with earlier stage of the disease. Based on the fact that oral mucosal lesions are easily detected clinically, oral premalignant lesions have been accepted as a largest tissue for preventing malignant transformations.

Epidemiological studies indicate that intervention at an early stage might reduce oral carcinoma related deaths. The discovery of immunological markers at a clinical, histological and molecular level has marked the end of
an era of groping in the dark for clues to the basis of cancer. Significant reduction in mortality can be achieved my advances in early diagnosis and implementation of multidisciplinary treatment programmes leading to improvement of survivorship and better quality of life.

**ORAL PRECANCER AND CANCER**

In India, oral cancer is prevalent in most areas where tobacco related practices are observed. For development of oral cancer, tobacco is the single greatest risk factor. This is due to higher concentration of carcinogenic exposure and failure to clean the carcinogens from the mucosal surface. If one observes the mouths of heavy tobacco users, the accumulation of tobacco residue maybe correlated with areas of oral cavity involved. Alcohol, viruses, genetic mechanisms, candida, chronic irritation and diet deficiency states are also implicated in the etiology.

The development of oral cancer is a multistep process arising from pre-existing potentially malignant lesions. Leukoplakia is the most common precancer representing 85% of such lesions. Histologically, over 95% of oral cancers are squamous cell carcinomas. It has been suggested that a vast majority of oral squamous cell carcinomas in India arise from pre-existing Leukoplakia.

Likewise, the incidence of oral submucous fibrosis (OSMF) is increasing like an epidemic, targeting the younger generation. The etiology for OSMF is still obscure and a varied number of factors have been proposed. Of these, areca nut use is the most important and persistent finding in history taking.
ROLE OF CIRCULATING IMMUNE COMPLEXES

Intensive studies have documented the role of immune complexes as modulators of both cellular and humoral immune response. The occurrence of circulating immune complexes (CIC) as a marker for tumor burden and prognosis in the sera of patients with oral precancer and cancer is now well established. Recent advances in the fields of CIC, tumor progression, drug resistance, tumor cell heterogeneity and metastasis have resulted in a renewal interest in the development of non-specific immunotherapeutic modalities\textsuperscript{11}

The overall consensus is that only a small percentage of the detected CIC in vivo represent tumor associated antigens complexed with antibodies. The bulk of CIC most likely represent auto antibodies or the reaction to denatured self proteins, microbes, normal lymphocyte, antigens and nuclear antigens\textsuperscript{12}. Antigenic made up of CIC in cancer patients reflects the host’s immune response to a variety of often overlapping antigenic stimuli and hence paves way for further studies\textsuperscript{13}.

Trace elements have been extensively studied in recent years to assess whether they have any modifying effects in the etiology of cancer. Copper, iron and selenium are essential for numerous enzymes and therefore it is reasonable to assume that variations in serum level of these biochemical markers maybe associated with the pathogenesis of oral cancer. The importance of these elements in cancer was reported by Schwarz (1975)\textsuperscript{14} which opened the door for new diagnostic and therapeutic
endeavours in many areas of medicine and specifically in the areas of oncology. Immunological and biochemical alterations in the serum of such patients can help not only in the early diagnosis, appropriate treatment but also as indicators of prognosis, as the disease progresses.

MATERIALS AND METHODS

This study was carried out in Nair Hospital Dental College, Mumbai in association with Topiwala National Medical College and Bhabha Atomic Research Centre, Mumbai. Proforma for the study was prepared and informed consent was taken from the subjects and controls.

Clearance was obtained from the B.Y.L. Charitable Nair Hospital Ethical Committee (Nair hospital golden jubilee research foundation). Patients were selected randomly as per the inclusion criteria.

Thirty patients with OSMF/L and 30 patients with OSCC with histopathologically proven lesions were included in this study. For comparison thirty normal subjects were also selected. The age group of these patients ranged from 25-70 years. The symptoms and signs of the patients were evaluated, after thorough history taking.

Criteria for selection of subjects for the study:–

Selection: Simple Random Sampling

All patients were subjected to detailed history regarding diet and addiction. Signs and symptoms were evaluated after thorough history taking.

- Patients with any systemic disease were excluded
- Routine blood investigations and Erythrocyte sedimentation rate estimation was done to rule out any systemic involvement
- Subjects and controls with no history of any major past or present illness, drug intake were included in the study.
- Oral submucous fibrosis and leukoplakia and squamous cell carcinoma was confirmed on the basis of histopathological examination of biopsy sample.

**Clinical evaluation:**

Patients with **OSMF** were clinically evaluated on basis of

1) Measurement of mouth opening (the maximum distance between upper and lower central incisor teeth) normal = 3.5- 4.5cm

2) Measurement of tongue protrusion (measured in full mouth opening position and fully extended position of tongue, from mesial angle of the maxillary central incisor to the tip of the tongue) normal = above 5cm

3) Burning sensation and blanched appearance of oral mucosa.

Patients with **Leukoplakia** were clinically evaluated according to

Non-scrapable, white/ red & white patch with majority of subjects having a history of tobacco intake.

1) Size of the lesion- maximum diameter of lesion noted

2) Number and extent of lesion

3) Site of lesions

- the patients were advised to stop their habit, chewing/smoking and to maintain proper oral hygiene
Patients with **Squamous cell carcinoma** (Well differentiated, moderately differentiated and poorly differentiated) were included in the study. Cases of ulceroproliferative, exophytic lesions were considered. Lymph node involvement/fixity was present in some cases. History of tobacco and areca nut in majority of cases was found.

**PROFORMA OF STUDY**

Proforma for oral malignant and premalignant condition/lesion.

Reg. No.

I. **Biodata**

<table>
<thead>
<tr>
<th>Name</th>
<th>Case no</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>Sex</td>
</tr>
</tbody>
</table>

II. **Chief complaint**

Onset
Duration
Progress
Relevant history

III. **Personal history**

Marital status
Educational status
Socio-economic status

Habits:

<table>
<thead>
<tr>
<th>Frequency/ amount per day</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>in year</td>
</tr>
</tbody>
</table>
- Tobacco: *chewing
  *Placing in
  the mouth

- Betelnut chewing
- Pan chewing / Pan masala
- Smoking: Bidi/ Cigarette/ Pipe/ Hukka/ Chutta
- Alcohol consumption

IV. Family history

V. Past medical history (particularly for any pre-existant malignant lesion)

  Diabetes/ Hypertension/ Blood dyscrasias/ Tuberculosis
  Premalignant conditions: Syphilis/ Anemia, etc

VI. Past dental history

VII. Extra oral examination:

Lesions/ swellings

- Site
- Size and extension
- Borders
- Surface
- Surface temperature of lesion
- Consistency
- Relation to underlying structure: fixed/ free from other structures

Lymph nodes
- Site  
  a) Region involved  
  b) Side involved  
- Size  
- Number and condition: a) Solitary  
  b) Multiple * Discrete/ Fused  
- Consistency: a) Solitary  
  b) Multiple  
  *Soft *Firm *Hard *Stony hard  
- Pain: Tender/ Non tender/ Painful  
- Relation with the surrounding structures: Fixed/ Freely movable  

**Temporomandibular joint:** Normal/ Restricted/ Complete trismus  

**Any other abnormality:** Specify  

**VIII. Intra oral examination**  
- Oral hygiene  
- General condition of teeth  
- General condition of oral mucosa  
- General condition of gingiva  
- Chronic irritation factors in close vicinity of the malignant or pre-malignant lesion  
  i. Carious teeth/ roots  
  ii. Fractured teeth/ roots  
  iii. Fractured fillings/ sharp fillings/ overhanging margins  
  iv. Dental appliances
IX. Prealignant condition/ lesion

Condition/ lesion- site/ size/ extent/ description

X. Malignant lesions

- Site
- Size and extent
- Surface and surface texture
- Borders
- Consistency
- Relation of the base with the underlying structures: fixed/ movable

XI. Investigations

- Radiographs: intraoral/ extraoral
- Biopsy: Incisional/ Excisional/ Punch
- Routine haematological investigations
- Serum analysis of circulating immune complexes, copper, Iron and Selenium

XII. Follow-up

INFORMED CONSENT FORM FOR “SUBJECTS”

TITLE: “CIRCULATING IMMUNE COMPLEXES AND TRACE ELEMENTS (COPPER, IRON AND SELENIUM) AS MARKERS IN ORAL PRE CANCER AND CANCER: A RANDOMISED CLINICAL CONTROLLED TRIAL”

I ……………………………………… being of sound mind, do fully and freely give my consent to participate as a subject in the study entitled “CIRCULATING IMMUNE COMPLEXES AND TRACE ELEMENTS”
(COPPER, IRON AND SELENIUM) AS MARKERS IN ORAL PRE CANCER AND CANCER: A RANDOMISED CLINICAL CONTROLLED TRIAL” being conducted by Dr. ………………………………. under the guidance of Dr. ………………………………

The purpose of the study, the procedure of the study, the benefits, the foreseeable risks and complications involved have been explained to the language I understand and speak best.

I have also been informed of my right to withdraw from the study at any time without giving any reason for doing so.

My signature/ left thumb impression confirms my consent for participation in the study

Name of the volunteer- Name of witness-
Address- Address-
Signature/ Left thumb impression Signature/Left thumb impression
Date Date

INFORMED CONSENT FORM FOR “CONTROLS”

TITLE: “CIRCULATING IMMUNE COMPLEXES AND TRACE ELEMENTS (COPPER, IRON AND SELENIUM) AS MARKERS IN ORAL PRE CANCER AND CANCER : A RANDOMISED CLINICAL CONTROLLED TRIAL”

I ………………………………………. being of sound mind, do fully and freely give my consent to participate as a subject in the study entitled “CIRCULATING IMMUNE COMPLEXES AND TRACE ELEMENTS
(COPPER, IRON AND SELENIUM) AS MARKERS IN ORAL PRE CANCER AND CANCER: A RANDOMISED CLINICAL CONTROLLED TRIAL” being conducted by Dr. ___________________________ under the guidance of Dr. ___________________________.

The purpose of the study, the procedure of the study, the benefits, the foreseeable risks and complications involved have been explained to the language I understand and speak best.

I have also been informed of my right to withdraw from the study at any time without giving any reason for doing so.

My signature/ left thumb impression confirms my consent for participation in the study.

Name of the volunteer- Name of witness-

Address- Address-

Signature/ Left thumb impression Signature/Left thumb impression

Date Date

The following investigations were carried out in Serum obtained from 10 ml of various blood collected from the subjects –

1) Serum estimation of CICs using 3.75% Polyethylene Glycol 6000 (PEG) serum percpitation.¹⁹

2) Serum estimation of Copper (Cu) using the Colorimetric Oxaly Dihydrazide method.²⁰

3) Serum analysis of Iron (Fe) using colorimetric Dipyridyl method.²⁰
4) Differential Pulse Cathodic Stripping Voltametry to estimate serum selenium (Se).\textsuperscript{21}

**STATISTICAL METHODS**

The data was subjected to statistical analysis using the Chi Square Test, Standard Deviation, Student’s unpaired t-test, correlation, ANOVA and Linear Regression.

Groupwise comparison of gender in three groups—Chi square test applied.

ANOVA test applied for:

Groupwise comparison of circulating immune complexes, copper, iron and selenium.

Comparison of various variables among precancer & cancer groups—Unpaired t-test was applied. Correlation was ascertained using correlation regression analysis.

Linear regression analysis with type of lesion as dependent variable and parameters as independent variables was used to identify the best predictors among age, gender, CIC, serum copper, iron & selenium for the type of lesion.

**RESULTS AND DISCUSSION**

Research emphasizes the development of generalizations, principles or theories that will be helpful in the prediction of future occurrences.

The immunological abnormalities in patients with cancer in the head and neck appear to be more profound than those associated with cancers of the bronchus, breast, cervix, colon or bladder (Litchenstein et al).\textsuperscript{22} The immunoglobulin deposits may represent immune (antigen-antibody)
complexes, since circulating immune complexes have been detected in 75% of patients with head and neck carcinoma (Scully et al). \(^{23}\)

Majority of our study group consisted of males (66.67%) who had tobacco, areca nut chewing and associated habits. The mean age was higher in the patients suffering from oral carcinoma (p value 1.10E – 13). (Table No. 1). p<0.05 is considered statistically significant.

Gross et al\(^ {24}\) reported that ageing is associated with a decline in the cell mediated immunity which might predispose to oncogenesis. Circulating immune complexes have been implicated in autoimmune diseases, neoplastic diseases, infectious diseases caused by bacteria, viruses and parasites. Scully C, Barkas T. et al\(^ {25}\) evaluated the circulating immune complexes in patients with squamous cell carcinoma and found them significantly raised. Hoffken et al\(^ {26}\) concluded that the elevation of circulating immune complexes was attributed to change in the levels of complement fixing and non-complement fixing of tumour specific antibodies. This implied that it may be possible to monitor the malignant transformation of premalignant lesions. Also, emphasis should be laid on the detection of the antigenic component of the circulating immune complexes.

In the presence study the levels of CIC show a gradual increase in the precancer group and the cancer group is characterized by a marked increase in levels which is statistically significant (p value 5.67E – 08) (Table No. 1 Graph No. 1,4). From these results it can be hypothetized that CIC represent the host’s physiological and immunological defense response in eliciting specific antibodies upon exposure to most antigenic substances.
CIC deposition further leads to inflammation and tissue / cell damage. It also leads to suppression of cell mediated immunity and modulates the humoral response.

High levels of copper in areca nut, a major etiological factor in OSMF plays an initiating role in stimulation of fibrogenesis by up regulation of lysyl oxidase (Ma. RH et al)\textsuperscript{27} and thereby causing inhibition of degradation of collagen. The rise in serum copper may be due to increased turnover of ceruloplasmin (a copper carrying globulin with essential oxidase activity) (Jaydeep et al)\textsuperscript{28} in the serum of carcinoma patients. Varghese et al\textsuperscript{29} concluded a significant reduction in serum copper in oral cancer, OSMF and leukoplakia patients.

Serum levels of copper showed gradual increase from precancer to the cancer group as compared to normals which was statistically significant (p value 0.012) (Table No. 1, Graph No. 2,4).

Serum Iron levels are considered as biochemical indicators for nutritional assessment. Utilization of iron in collagen synthesis\textsuperscript{30} by the hydroxylation of proline and lysine leads to decreased serum iron levels in OSMF patients. In most cases clinical anemia may be a contributing factor. (Ramanathan et al).\textsuperscript{31}

A statistically significant reduction in the serum iron level was present in the precancer group in our study. A decrease in the iron levels in the cancer group, but higher than that of pre cancer groups was found to be significant. (p value 2.35E – 19) (Table No. 1, Graph No. 3,4)
There appears to be an association between the serum iron content and oral carcinogenesis. More detailed studies on a large data base should be instituted to elucidate the exact role of iron.

Selenium forms the integral part of the enzyme glutathione peroxidase, type I iodothyronine deiodinase, metalloprotein, fatty acid binding protein and selenoprotein P. therefore selenium is considered as an antioxidant nutrient and the diseases where low selenium is implicated range from nutritional disorders like protein energy malnutrition to degenerative diseases such as cancer\textsuperscript{32}.

Vijaykumar T reported an increase in serum selenium in oral leukoplakia and oral cancer\textsuperscript{33}. Various epidemiological studies have implicated selenium as a cancer protective agent. Studies indicate that higher dietary intake of selenium in humans may be protective.

The serum selenium concentration was found to be decreased (p value 0.187). The role of selenium is thus complex which can be attributed to its protective antioxidant role. (Table No. 1, Graph No. 4).

A significant positive correlation as present between the serum circulating immune complexes levels and copper in the pre cancer group. Both parameters showed a steady increased (Graph No. 5). there was a significant positive correlation was found between age of subjects and circulating immune complexes. A significant correlation was also noted in serum copper and iron levels in the precancer group (Graph No. 6).

Linear regression estimates the coefficient of the linear equation involving one or more independent variables that best predict the value of
the dependent variable. Applying linear regression analysis with type of lesions as dependent variable, we identified age, serum iron, CIC and serum levels of selenium as best predictors for the occurrence and progression of lesions in the decreasing order (Table No. 2). However, gender and serum copper failed to show any predictive value for the type of lesion. Estimation of CIC and trace elements might help in early detection, differential diagnosis and treatment planning of oral premalignant and malignant lesions.

CONCLUSIONS

The present study highlights that circulating immune complexes represent the host’s physiological and immunological response in eliciting specific antibodies upon exposure to most antigenic substance.

High levels of copper in areca nuts, a major etiological factor in OSMF plays an initiating role in stimulation of fibrinogenesis by up regulation of lysyl oxidase and thereby causing inhibition of degradation of collagen and causing its accumulation thereby causing OSMF. The rise in serum copper may be due to increased turn over of ceruloplasmin in the serum of carcinoma patients.

Serum iron levels are considered as biochemical indicators for nutritional assessment. Utilization of iron in collagen synthesis by the hydroxylation of proline and lysine leads to decrease serum iron levels in OSMF patients. In most cases clinical anemia may be a contributing factor. Inadequate intake of food due to burning sensation and vesiculation in the oral cavity might also be an important factor. Reduction in the serum iron
level may be due to malnutrition caused by the tumor burden in cancer patients.

A decrease in the serum selenium level in oral carcinoma patients can be attributed to the protective antioxidant role in cancer. No similar study has been done on serum levels of circulating immune complexes, trace elements, (copper, iron and selenium) as a combination in oral precancer and cancer.

An attempt was made to assess these parameters as predictors for disease occurrence and progression. We identified age, serum iron, CIC and serum levels of selenium as best predictors for the occurrence and progression of lesions in the decreasing order.

It can be suggested that immunological and biochemical assessment of oral precancer and cancer patients may help in earlier diagnosis and/or prognosis of these lesions. This may also serve in predicting malignant potential of the pre malignant lesions.

These efforts maybe of value for proactive intervention of high risk groups. (potentially malignant conditions and lesions)

*Proactive intervention might be an inconvenience,*

*But the decision is ours,*

*An inconvenience rightly considered,*

*Or a convenience wrongly considered.*
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Nair Hospital Dental College, Mumbai.

REFERENCES

1) Daftary DK, Murti PR, Bhonsle RR et. al : Risk factors and risk areas of the
   world. In Johnson NW and cancer the detection of patients and lesions at

2) Shah J., Johsnon N, Batasakis J : Global epidemiology, oral cancer, Martin
   Duntiz Group 2003,3.

3) Quarri D, Adams G et al Head & Neck Cancer : Clinical decisions and
   management principles, 1977; 219-220.

4) Deshpande VA and Jussawalla DJ:Evaluation of cancer risk in tobacco
   chewers and smokers : An epidemiological assessment. Cancer,
   1971;28:244-252

5) Gupta PC, Mehta F.S. et al :Comparison of carcinogenicity of Betel quid

6) Bonquot JE, Whitaker SB: Oral Leukoplakia rationale for diagnosis and
   prognosis of its clinical subtypes or “phases”. Quaintessence Int
   1994;25:133-140.


LEGENDS

Table no 1: Groupwise comparison of various variables among all cases

Table No 2: Linear Regression Analysis with type of lesions as dependant variable.

Graph No 1: Illustrates marked increase in levels of CIC in precancer (OSMF/L) and cancer groups.

Graph No 2: Gradual increase of copper levels from precancer to cancer as compared to normal.

Graph No 3: Indicates statistically significant reduction in the serum iron levels of precancer and cancer group as compared to normal.

Graph No 4: Groupwise comparison of CIC, copper, iron and selenium.

Graph No 5: Correlation between CIC and copper in precancer group.

Graph No 6: Correlation between copper and iron in precancer group.

Competing interests: the authors have no competing interests

Authors contribution:

Dr Sunali Khanna carried out the study, performed biopsies, participated in design, sequence alignment, drafted and edited the manuscript.

Dr Freny Karjodkar is the incharge H.O.D. of the dept where the study was carried out under her guidance.