MATERIAL AND METHODS

Studies were conducted to evaluate the possible correlation of smoking and tobacco chewing with S-IgA concentration from unstimulated whole saliva. This Chapter describes the details of study design, selection of subjects and methodology used.

(1) Selection of Patients/Subjects

Healthy adult males attending Out Patient Department, Government Dental College and Hospital, Ahmedabad, aged between 16 and 35 years, were selected after obtaining their consent to participate in the present study (Shah, 1990). Patients included in the study belonged to good socio-economic status and at least secondary educational level i.e. Matriculation. Detailed history was recorded on frequency and duration of smoking and tobacco chewing. Special care was taken to ensure that most of the selected subjects had started the habit of using tobacco in their adolescent age. Subjects selected for tobacco chewing group were those consuming commercially available tobacco pouches usually containing pre-prepared mixture of tobacco, lime, arecanut, catechu principally in fixed quantity and quality.

The following specific criteria were used for selection of subjects for the present study.
Group I (Tobacco Smokers):
- Continuous history of smoking regularly, filter tipped, non-mentholated, commercially available cigarettes at least for the last three years. The major exclusion criteria was that they should no history of usage of tobacco toothpaste, arecanut, snuff, chutta, mawa, khaini 'pan masala' or bidi.

Group II (Tobacco Chewers):
- Continuous history of chewing commercially available tobacco pouches at least for the last 3 years. The major exclusion criteria was absence of habit of other forms of smoking or smokeless tobacco including 'pan', 'quid', tobacco toothpaste, etc.

General inclusion criteria for both Groups:
(i) Full set of teeth excluding third molars.
(ii) Evidence of fair oral hygiene practices with use of toothbrush, toothpaste and tongue cleaner regularly.
(iii) No history of prophylaxis treatment through dentist for at least two years.

General exclusion criteria for both Groups:
(i) No history of alcohol consumption (Mandel et al., 1979; Bennet and Reade, 1982)
(ii) Not addicted to drug(s).
(iii) Not taking any medicines regularly which may modify the salivary flow rates (Mandel et al., 1979; Bennet and Reade, 1982)

(iv) No history of upper respiratory disease of acute, chronic or infectious origin.

(v) Frank neoplastic ulcer cases.

(vi) Subjects having allergic or auto-immune disorders.

(vii) No history of medicines that alter the immune response.

(viii) No history of debilitating disease and exanthematous fevers during the past 6 months.

(ix) No history of diabetes mellitus (metabolic disorders), essential hypertension or ischaemic heart disease.

(x) No occupational exposure to tobacco.

(xi) No overt salivary gland dysfunction (Mandel et al., 1979; Bennet and Reade, 1982).

The selected subjects were divided into the following sub-groups.

(2) Control Group:

Healthy adult males between 16 and 35 years of age who had never smoked cigarette or chewed arecanut containing pan were recruited as controls. They were hailing from comparable socio-economic strata and educational level of test
subjects. The aforementioned general inclusion and exclusion criteria were also considered in selecting the controls. They served as controls to all smoking and chewing sub-groups.

TERMINOLOGY:

**Cigarette years (smoking intensity index):** Smoking exposure can be expressed in terms of cigarette years (C.Y.) (IARC., 1986)

\[ C.Y. = \text{number of cigarettes smoked a day} \times \text{number of years the subject had been smoking}. \]

**Smokeless Tobacco hours/day (chewing intensity index):** The number of hours the chewing tobacco was held in the mouth at a particular site in lower jaw/vestibule per day was calculated as combined measure of intensity of use (Daniel et al., 1990).

\[ \text{Smokeless Tobacco hours in mouth/day} = \]

\[ \text{No. of pouches used per week} \times \text{No. of chews/dips per pouch} \times \text{No. of minutes each dip/chew is held in mouth} \]

\[ \div 420 \ (60 \text{ min.per hour} \times 7 \text{ days per week}) \]

Based on self-reported smoking and chewing use, the subjects were classified as light, moderate and heavy users.

In representative sample of light smokers and light chewers, biochemical validation of self-reported tobacco usage was carried out with serum cotinine and serum thiocynate estimations as per techniques described by Haley et al (1983). Since serum thiocynate is elevated only in cigarette smokers, elevated serum cotinine in the presence of normal serum
thiocynate was considered to be a biochemical evidence of habitual chewing (Haley et al., 1983; WHO., 1988).

(3) **Sub-Groups of Smokers and Chewers**

Following is the list of subgroups of smokers and chewers with relevant criteria for their inclusion in the present study.

(A) **SMOKERS**

(a) **Light smokers:**

The selected subjects had smoking exposure between 10 and 150 cigarette years.

(b) **Moderate smokers:**

The selected subjects had smoking exposure between 165 and 297 cigarette years.

(c) **Heavy smokers:**

The selected subjects had exposure between 300 and 720 cigarette years.

(d) **Passive Smokers group:**

The subjects selected in this group were spouses, i.e. wives of compulsive smokers who occasionally/generally smoked in front of them leading to side-stream exposure of cigarette smoke known as "passive smoking" (WHO, 1990). The age of the husbands ranged between 26 and 35 years, with smoking exposure between 140 and 640 cigarette years. The wives (spouses) aged between 25 and 32
years, with 'passive' smoking exposure between 25 and 300 cigarette years. The exposure of the spouses was calculated taking into account the number of cigarettes smoked in a day by their husbands in their presence/vicinity and the number of years they had been experiencing this exposure.

(e) **Total smokers:**
   It included all the subjects of light, moderate and heavy smoker sub-groups with smoking exposure between 10 and 720 cigarette years.

(B) **CHEWERS**

(a) **Light chewers:**
   Selected subjects had chewing exposure between 1.0 and 2.8 hrs/day.

(b) **Moderate chewers:**
   Selected subjects had chewing exposure between 3.1 and 5.9 hrs/day.

(c) **Heavy chewers:**
   Selected subjects had chewing exposure between 7.5 and 11.00 hrs/day.

(d) **Total chewers:**
   It included all subjects of light, moderate and heavy chewers with chewing exposure between 1.00 and 11.00 hrs/day.
(C) SMOKER & CHEWER

Most of the subjects selected in this group were initially smokers, but with commercial availability of tobacco pouches, in addition to smoking they also started the habit of tobacco chewing. Their age ranged between 27 and 35 years, with smoking exposure between 140 and 425 cigarette years, and chewing exposure between 3.3 and 9.0 hrs/day.

SMOKING DETERRENT TABLETS:

BANTRON® Smoking Deterrent Tablets, (JMI-DEP Corporation, CA 90220, U.S.A.), were used as aids in breaking tobacco habit of individuals pharmacologically. It had convenient t.i.d. dosage after meals (i.e. thrice daily), to be continued for no longer than 6 weeks. Following was the chemical composition of each tablet:

(1) Lobeline Sulfate 2 mg
(2) Tribasic Calcium Phosphate 129.6 mg
(3) Magnesium Carbonate 129.6 mg.

(1) Lobeline Sulfate - It is an active ingredient. It has weak nicotine-like action on peripheral and central nervous system (Gilman et al., 1991). It is absorbed from the gastrointestinal (g.i.) tract immediately, and the serum levels remain for 6 to 8 hours. It is excreted through urine. It has side effects like severe gastric distress, heart burns, diarrhoea, etc. Symptoms of overdose include profuse diaphoresis, paresis, tachycardia, hypothermia, hypertension and coma. Fatalities have
occurred too. It is also included in preparations aimed at relieving bronchial asthma and chronic bronchitis. 

(2)\&(3) - Tribasic Calcium Phosphate and Magnesium Carbonate - These are passive ingredients generally included in tablets to counteract gastric irritation by Lobeline as they are strong antacids (Gilman et al., 1991).

**EVALUATION OF IMMUNE RECOVERY**

The selected subjects in this group were either smokers or chewers with variable smoking or chewing exposure. The selected subjects were aged between 19 and 35 years, with smoking exposure between 12 and 510 cigarette years. The subjects selected for chewer group aged between 19.5 and 34 years, with chewing exposure between 1 and 8.8 hrs/day. All of them had very high motivation to quit tobacco but could not do so. The test subjects were given both behavioural and pharmacological treatment in form of t.i.d. dosage of Lobeline sulfate 2 mg (thrice daily). Before recruiting the individuals in the quitting programme, the selected subjects underwent salivary IgA estimation protocol. Once taken into the quitting programme the subjects were examined biochemically for their 'non-user of tobacco' status repeatedly during the first month and then randomly during the following six months. At the end of six months S-IgA estimation was done. This procedure was repeated at the end of one year. The tobacco deterrent tablets were discontinued by 45th day by gradually tapering the dosage to prevent addiction to Lobeline sulfate compound, if any. The
selected subjects reported once in every fortnight for collection of tobacco deterrent tablets. Simultaneously behavioural/psychotherapy strategies were continued during the entire one year period, to help in checking the recovery in depleted levels of S-IgA.

EVALUATION OF LEUKOPLAKIA REVERSAL AND OBSERVED S-IgA ALTERATION

The selected subjects in this group were either smokers or chewers with high to very high smoking and chewing exposure. They all had presence of clinically detectable white lesions over oral mucosa, i.e. Leukoplakia. Leukoplakia, for the purpose of the present study, was defined as any white lesion that did not rub off and was not identifiable clinically as some other white lesion (Axell et al., 1984; Daniel et al., 1990; Little et al., 1992). Frictional keratoses, interdental hyperkeratosis, cheek biting and retromolar hyper keratosis were excluded from defining leukoplakia for the present study.

Any lesion classified as 'leukoplakia' was subsequently defined by the size in millimetre (mm), location in mouth, adjacent/non-adjacent to site of placement of tobacco preparation, colour (normal, white, red or red and white), texture (smooth, granular and corrugated), contour (raised, falt or cratered) and degree of severity on a scale from 1 to 4, as follows:

1 = No, or only slight colour change with a texture change.
2 = Colour and texture change, but no thickening.
3 = Colour and texture change with nil to moderate thickening.
4 = No normal colour, severe texture change, heavy thickening/cratering.

Subjects with more than one leukoplakia were classified by the lesion of the highest degree for analysis.

These subjects were made aware of the presence of premalignant lesion over their mucosa, in addition to restricted opening of mouth (submucous fibrosis) if present, to motivate them to quit tobacco. They were also explained about the chances of conversion of premalignant lesion to frank malignancy and only those with high motivation to quit tobacco were recruited in this group. The selected smoker subjects in this group ranged between 28 and 35 years of age with smoking exposure between 110 and 510 cigarette years. The chewer subjects were aged between 21 and 34 years, with chewing exposure between 1.5 and 8.8 hrs/day. The severity of leukoplakia grades varied between 1 and 3 in both the subgroup of smokers and chewers. Prior to recruiting in the quitting programme, these subjects underwent the S-IgA estimation protocol and grading of leukoplakia. Once included in the quitting programme, they were checked biochemically for their 'non-user of tobacco' status repeatedly for the first month and then randomly during the following six months. At the end of six months S-IgA estimation and grading of leukoplakia was done. The same procedure was repeated at the end of 1 year.
with random biochemical validation of 'non-user of tobacco' status during next six months also.

**DETERMINATION OF ORAL HYGIENE STATUS**

The oral hygiene status of the selected subjects in various smoker and chewer subgroups was determined by using oral hygiene index (Green, 1960) comprising of debris index and calculus index. The subjects were examined on a Dental chair under artificial light using mouth mirror, explorer and curved probe.

(a) **Debris Index**: (Green, 1960). It was used for the evaluation of the extent of debris present on the individual tooth surface, i.e. mesial, distal, buccal and lingual surfaces of randomly selected quadrant of teeth. The surface area covered by debris was estimated by running explorer on each tooth surface.

**Criteria for Debris Index**

<table>
<thead>
<tr>
<th>Score</th>
<th>Criteria and Scoring for Field Studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Absence of debris</td>
</tr>
<tr>
<td>1</td>
<td>Soft debris covering not more than cervical one-third of tooth surface.</td>
</tr>
<tr>
<td>2</td>
<td>Soft debris covering more than cervical one-third of the exposed tooth surface.</td>
</tr>
<tr>
<td>3</td>
<td>Soft debris covering more than cervical two-third of the exposed tooth surface.</td>
</tr>
</tbody>
</table>
(b) Calculus Index: (Green, 1960). An explorer was used for scoring of calculus. The surface area covered by calculus was detected supragingivally and subgingival calculus was explored for randomly selected quadrant of teeth.

Criteria for Scoring Calculus Index

<table>
<thead>
<tr>
<th>Score</th>
<th>Criteria and Scoring for Field Studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Absence of calculus</td>
</tr>
<tr>
<td>1</td>
<td>Calculus covering not more than cervical one-third of the exposed tooth surface was examined.</td>
</tr>
<tr>
<td>2</td>
<td>Supragingival calculus covering more than cervical one-third, but not more than cervical two-third of the exposed tooth surface, was examined, or presence of individual flecks of subgingival calculus around the cervical portion of tooth.</td>
</tr>
<tr>
<td>3</td>
<td>Supragingival calculus covering more than cervical two-third of the exposed tooth surface, or a continuous heavy band of subgingival calculus around the cervical portion of tooth.</td>
</tr>
</tbody>
</table>

DETERMINATION OF PERIODONTAL DISEASE STATUS

Periodontal disease status of selected subjects in various smoker and chewer subgroups was determined by using Gingival Index (Loe and Sillness, 1963), and Periodontal Index (Russell, 1957). The subjects were examined on a Dental chair under artificial light using mouth mirror, explorer and periodontal probe.
(a) **Gingival Index**: (Loe and Sillness, 1963). This index was used to determine the extent of inflammatory status of marginal and attached gingiva. All individual tooth surfaces of randomly selected quadrant of teeth were explored to achieve the index score.

**Criteria for Gingival Index**

<table>
<thead>
<tr>
<th>Score</th>
<th>Criteria and Scoring for Field Studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal gingiva</td>
</tr>
<tr>
<td>1</td>
<td>Mild inflammation - Slight change in colour and oedema. There is no evidence of bleeding on probing.</td>
</tr>
<tr>
<td>2</td>
<td>Moderate inflammation - Redness, oedema, glazing and gingival bleeding on probing.</td>
</tr>
<tr>
<td>3</td>
<td>Severe inflammation - Marked redness, oedema and gingiva showing tendency to spontaneous bleeding.</td>
</tr>
</tbody>
</table>

(b) **Periodontal Index**: (Russell, 1957). This index was used to assess the extent of periodontal destruction, pocketing and loss of alveolar bone in randomly selected quadrant of teeth.

**Criteria for Periodontal Index**

<table>
<thead>
<tr>
<th>Score</th>
<th>Criteria and scoring for Field Studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Negative - There is neither overt inflammation in the investing tissue nor loss of function due to destruction of supporting tissue.</td>
</tr>
</tbody>
</table>
Mild gingivitis - There is an overt area of inflammation in the free gingiva, but this area does not circumscribe the tooth.

Gingivitis - Inflammation completely circumscribes the tooth, but there is no apparent break in epithelial attachment.

Gingivitis with pocket formation - The epithelial attachment has been broken and there is a pocket (not merely a deepened gingival crevice due to swelling in the free gingivae). There is no interference with normal masticatory function. The tooth is firm in its socket and has not drifted.

Advanced destruction with loss of masticatory function - The tooth may be loose, may have drifted, may sound dull on percussion with metallic instrument, may be depressible in its socket.

Rule: When in doubt, assign the lower score.

---

EXAMINATION OF TEETH

It was divided as follows:

(a) Occlusal attrition, and
(b) Extrinsic tobacco stains

(a) Attrition index: (Ramfjord, 1947). Attrition, i.e. functional wear of tooth was determined on randomly selected quadrant of teeth after drying them.

Criteria for Attrition Index

<table>
<thead>
<tr>
<th>Score</th>
<th>Criteria and Scoring for Field Studies</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal anatomy of occlusal surface is present without destruction of any cusp.</td>
</tr>
</tbody>
</table>
1. Destruction of cusp is limited to enamel which can be seen transparent clinically.

2. Destruction of cusp also involves the dentin which looks opaque and dark yellowish in colour.

3. Extensive destruction of the cusp so that there will be a pulp exposure which shows dark brown colour clinically.

(b) **Stains Index** (Pathak, 1978). The following criteria were evolved after a pilot study for scoring of stains on randomly selected quadrant of teeth.

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<tr>
<td>0</td>
<td>Absence of stains</td>
</tr>
<tr>
<td>1</td>
<td>Stains covering one-third of exposed tooth surface.</td>
</tr>
<tr>
<td>2</td>
<td>Stains covering the tooth surface more than cervical one-third, but not more than cervical two-third portion of the tooth.</td>
</tr>
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<td>3</td>
<td>Stains covering more than cervical two-third of exposed tooth surface.</td>
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**GINGIVAL RECESSION INDEX** (Miller, 1985):

This index was used specifically to determine the extent of gingival recession, i.e. displacement of the gingival margin at least 1 mm apical to the cemento-enamel junction in
chewer subgroups at the site of placement of tobacco preparation.

Criteria for Gingival Recession

<table>
<thead>
<tr>
<th>Score</th>
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</tr>
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<tbody>
<tr>
<td>Class-I</td>
<td>This includes marginal tissue recession that does not extend to the mucogingival junction. There is no loss of bone or soft tissue in interdental area.</td>
</tr>
<tr>
<td>Class-II</td>
<td>Marginal tissue recession that extends to or beyond the mucogingival junction. There is no loss of bone or soft tissue in the interdental area.</td>
</tr>
<tr>
<td>Class-III</td>
<td>Marginal tissue recession that extends to or beyond the mucogingival junction; in addition, there is bone and/or soft tissue loss interdentally, or there is malpositioning of tooth.</td>
</tr>
<tr>
<td>Class-IV</td>
<td>Marginal tissue recession that extends to or beyond the mucogingival junction with severe bone loss and soft tissue loss interdentally and/or severe tooth malposition.</td>
</tr>
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RECORDING OF DATA

For systematic and methodical recording of the data without errors of transcription, a special Proforma was designed. The investigator himself interviewed all the subjects and recorded the details carefully. The salivary samples from test subjects and controls were coded following "double blind" method.
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**Criteria for Gingival Recession**

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<td>Class-IV</td>
<td>Marginal tissue recession that extends to or beyond the mucogingival junction with severe bone loss and soft tissue loss interdentally and/or severe tooth malposition.</td>
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</table>

**RECORDING OF DATA**

For systematic and methodical recording of the data without errors of transcription, a special Proforma was designed. The investigator himself interviewed all the subjects and recorded the details carefully. The salivary samples from test subjects and controls were coded following "double blind" method.
The patients selected for S-IgA analysis were called on a particular day on empty stomach at 9.00 a.m. to the Dental College, Department of Periodontia, Ahmedabad. The patient was advised to have his oral hygiene measures completed the previous night. He was asked to refrain from spitting, oral hygiene measures and smoking/chewing thereafter. The patient was given 50 ml. of ultra-pure distilled water (Laboratory Grade) to gargle for 30 sec. in oral cavity with normal force. 25 sec. was used as actual gargling time and the remaining 5 sec. to empty "whole saliva" gently into the pre-sterilized glass bottle through a funnel. 25 ml of the ultra-pure distilled water was poured into the funnel circumferentially to wash out the proteins adhering on the funnel walls. The sample was then immediately transported to the laboratory for ordinary centrifuge. Salivary samples were centrifuged using clinical centrifuge at 3000 rpm for 10-15 min., thereby the solids at the bottom were discarded and the supernatent thus collected was stored between -20°C and -135°C in Cryogenic Preservation System of Queue Cryostar, U.S.A.

LYOPHILIZATION PROCESS

20 ml. of the stored supernatent was freeze-dried in Lyophilizer (Model 3100 BULT, Refrigeration for Science Inc., NY, U.S.A.). Salivary sample underwent manifold drying at -80°C under vacuum pressure below 100 millitore for 8-10 h. This process dried most of the liquid present in the sample,
COLLECTION AND STORAGE OF SALIVA

The patients selected for S-IgA analysis were called on a particular day on empty stomach at 9.00 a.m. to the Dental College, Department of Periodontia, Ahmedabad. The patient was advised to have his oral hygiene measures completed the previous night. He was asked to refrain from spitting, oral hygiene measures and smoking/chewing thereafter. The patient was given 50 ml. of ultra-pure distilled water (Laboratory Grade) to gargle for 30 sec. in oral cavity with normal force. 25 sec. was used as actual gargling time and the remaining 5 sec. to empty "whole saliva" gently into the pre-sterilized glass bottle through a funnel. 25 ml of the ultra-pure distilled water was poured into the funnel circumferentially to wash out the proteins adhering on the funnel walls. The sample was then immediately transported to the laboratory for ordinary centrifuge. Salivary samples were centrifuged using clinical centrifuge at 3000 rpm for 10-15 min., thereby the solids at the bottom were discarded and the supernatent thus collected was stored between -20°C and -135°C in Cryogenic Preservation System of Queue Cryostar, U.S.A.

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This process dried most of the liquid present in the sample,
leaving the powder which was measured on dry-weight basis to achieve the level of concentration, considering the concentration gradient of 1:100 times of original volume with normal saline.

**S-IgA ASSAY**

"Solugen®" plain SRID plates (Immunodiagnostics Pvt.Ltd., Delhi-110006, India) were used for analysis of S-IgA. These plates are based on Radial Immunodiffusion Technique (Mancini et al. 1965). The sample (5μl) was charged with the help of micropipettes into the well of the plates. The plates were kept for incubation in humid chamber for 50 h. The diameter of precipitation ring (Ag-Ab reaction) was measured after 50 h. using Immunomeasure® supplied by the manufacturer. After referring to the linear to log scale, the S-IgA level was obtained.

**STATISTICAL METHOD**

Mean and Standard Deviation (S.D.) of all the indices were calculated for all subgroups of smokers and chewers separately alongwith control group. All groups were compared with control to see the difference in S-IgA level by Students 't' test. Paired 't' test was applied to achieve the level of significance in intra-group and inter-group comparison of various indices. For all subgroups of chewers, 'Chi-square' test was applied to bring out significant linearity of recession. Mean and Standard Deviation of salivary S-IgA levels were obtained for all subgroups of smokers and chewers.
to be compared with control by Students 't' test. Intra-group and Inter-group comparison was done by paired 't' test. Regression analysis was carried out and regression equations obtained for various subgroups of smokers and chewers for predicting S-IgA in ex-smoker and ex-chewer at the time of quitting tobacco habit. Regression equations were obtained after multivariate analysis of Total Smokers (light smokers + moderate smokers + heavy smokers) and Total Chewers (light chewers + moderate chewers + heavy chewers). 'Chi-square' test was applied to bring out remission of leukoplakia severity on tobacco cessation at 6 months and 1 year.