Materials
And
Methods
High incidence of oral cancer in this part of the world is believed to be associated with chewing of tobacco and areca nut, prevalent in this region. The chewing quid remains in close contact with oral mucosa during mastication. Thus, there is a need to study the cytogenetic changes in oral mucosa using cytome assay, which might serve as an early biomarker of oral lesions. In addition, cytogenetic endpoints in the peripheral blood lymphocytes of chewers and non-chewers were determined using chromosomal aberrations and cytokinesis block micronucleus assay. It is important to know the involvement of genetic damage in the causation of oral lesions along with other factors. Blood cells DNA damage was also studied by using the comet assay and cotinine (metabolite of nicotine) level was measured in blood plasma of chewers and non-chewers. In addition, copper and zinc levels in serum were studied to find out any relationship between these metals with the changes in oral cavity. Structural and numerical chromosome 17 abnormalities in blood cell of OSMF and areca nut and tobacco chewers were investigated by labeling 17cen and the p53 locus by multicolour FISH that allows the efficient detection of 17p deletions leading to p53 deletions, 17p gains and whole chromosome 17 numerical abnormalities in cultured blood cells.

**Study setting:** This study was undertaken in Government Dental College and Hospital, Civil Hospital Campus, Ahmedabad, Gujarat, India.

**Sample size:** The sample size was determined on the basis of the previous study of Adhvaryu *et al.*, (1991). The average chromosomal aberration levels
in control population were 5.0 (unit) with standard deviation of 1.549. As we have registered the patients from the dental hospital for check up, the same values were considered with margin of error to be 0.5, with usual constant \( \alpha=0.01, \beta=0.01 \) and power of study 99%. The sample size for the study was found to be approximately two hundred thirty one.

1. **Selection of the subjects:** A cross sectional study was conducted among apparently healthy subjects who were attending the Out Patient Department (OPD) of Government Dental College and Hospital for various dental diseases. The subjects were enrolled and classified as chewers (according to chewing habits), Oral submucous fibrosis subjects and non-chewers. Among chewers, subjects were further categorized on the basis of chewing habits such as areca nut with tobacco, areca nut without tobacco and only tobacco chewers. Further, the chewing exposure was calculated in terms of the frequency of quids of areca nut/tobacco used per day multiplied by the duration of chewing exposure in years, which was referred to as the Lifetime chewing exposure (LCE) (Beena *et al.*, 2009).

1.1 **Inclusion criteria:** The following criteria were used for inclusion of the study subjects:

- No history of drug addiction.
- Not taking any medicines regularly.
- No history of upper respiratory disease of acute, chronic or infectious origin.
- No history of diabetes mellitus, essential hypertension or ischemic heart disease.
- No known history of psychological disorder.

1.2 **Informed Consent:** The objectives as well as the benefits of the study to individual and society as a whole were explained to the study subjects. They were also informed about the ill effects of chewing tobacco and areca nut and a written consent to participate in study was obtained.
1.3 **Ethical clearance:** The ethical clearance of the study was taken from the Institutional Ethics Committee of National Institute of Occupational Health, Ahmedabad chaired by Mr. S.D. Dave, ex-Hon’ble Chief Justice of Gujarat High Court.

2. **Questionnaire survey:** A specific proforma was designed with the assistance of epidemiologist and bio-statistician (Appendix-II). The same was pre-tested and necessary modifications were made accordingly. The following salient information was collected and recorded by administering the questionnaire to the subjects.

1) Personal information comprising i.e. name, address, age, sex, occupation, educational status, annual income etc.

2) Awareness about the possible ill effects of chewing habits.

3) Detailed history of chewing habits — material(s) used e.g. areca nut alone, areca nut with lime and tobacco *(mawa)*, tobacco alone, pan masala plain or pan masala with tobacco *(gutkha)*, pan, *khaini* etc.

4) Duration of chewing habit in years, number of quids chewed per day, site of placement was recorded.

5) Details of smoking and drinking habit along with the duration and frequency.

3. **Clinical observations:** Following, clinical observations were carried out under the guidance of clinicians by adopting various standard criteria/methods:

3.1 **Oral Hygiene status has been undertaken**

3.2 **Measurement of mouth opening:** The subjects were asked to open the mouth to their maximum and measurement of distance between the upper and lower incisors and molars (mm) were carried out using vernier caliper.
3.3 Oral soft tissue lesions: Clinical examination of oral cavity was carried out using curved probe, mouth mirror and torch light to observe the clinical changes, if any, in oral cavity of all the subjects. The oral cavity was checked for oral mucosal lesion i.e. submucous fibrosis, leukoplakia, lichenplanus and ulcer etc. The salient clinical features of these lesions, are as follows:

Oral submucous fibrosis was graded into three stages according to clinical signs and symptoms (Bhatt and Dholakia, 1977).

**Mild** - Very slight fibrous banding and little narrowing of mouth opening

**Moderate** - Moderately pronounced symptoms, fibrous bands extending from cheek to palate

**Severe** - Excessive amount of fibrous banding involving cheeks, palate, uvula, tongue and lips and marked narrowing of mouth opening.

WHO (1978) criteria were adopted for the diagnosis of leukoplakia. Leukoplakia is a raised white patch of the oral mucosa measuring 5 mm or more, which cannot be scrapped off and cannot be attributed clinically or pathologically to any other diagnosable disease. On clinical examination, patches of leukoplakia may vary from a non-palpable, faintly translucent white area to thick, fissured, papillomatous, indurated lesions. An intermediate stage consists of localized or diffuse, slightly elevated plaques of irregular outline, which are opaque, white and may have a fine granular texture. The surface of the lesion is often finely wrinkled or shriveled in appearance and may feel rough on palpation. The lesions are white, gray or yellowish-white but, with heavy use of tobacco, may assume a brownish-yellow colour.

The appearance of tobacco-quid lesion varies from superficial lesion with a colour similar to the mucosa with slight wrinkling and no obvious thickening to a marked white or yellowish to brown and heavily wrinkled lesion with intervening deep and reddened furrows and or heavy thickening (Zain, 1999).
4. Collection of biological materials: Both blood as well as buccal mucosa cells were collected between 09:30 a.m. and 11:30 a.m.

4.1 Collection of blood sample:
About six milliliters of blood was collected from the medial cubital vein using a vacutainer. A part of whole blood (approx 3 ml) was collected in heparinised vacutainer for \textit{in vitro} culture and plasma collection. Each sample was mixed gently to avoid clotting and kept at 37°C till the time of culture setting. Cultures were setup within two hrs of sample collection. Approximately 3 ml of blood sample was collected into an anticoagulant free tube. After allowing about 60 min for spontaneous blood clotting, the serum was separated by centrifugation (REMI, India) at 3000 rpm for 10 min at room temperature. Similarly plasma was separated from heparinised blood by centrifugation at 3,000 rpm for 10 min at room temperature. The serum and plasma was stored in a metal free polypropylene tube at –20°C deep freeze (REMI, India).

4.2 Collection of buccal mucosal cell:
Before the collection of buccal mucosa cells, subjects were asked to rinse the mouth thoroughly with tap water. The exfoliated buccal mucosa cells were scrapped briskly using wooden spatula. These cells were transferred into the PBS buffer and store at 4°C for buccal cytome assay.

5. Detailed Examination of biological materials:

5.1 Preparation of culture media and reagents: Most the chemicals were procured from Sigma (Aldrich, USA).

\textbf{Stock preparation RPMI medium:}
RPMI-1640 medium with L- Glutamine, lyophilized powder was dissolved in 900 ml autoclaved triple distilled water in which 2 gm NaHCO$_3$, 10 mg streptomycin and 1000 U of penicillin was added, mixed thoroughly on a magnetic stirrer and then set pH 7 with 0.1 N HCl. Make up the volume to 1000 ml. Filter with vacuum filtration assembly. The above mixture was first
filtered through whatman filter paper no.1 and then through millipore filter having 0.22 μm porosity. After dispensing the medium in 100 ml sterile bottle, it was stored in at 4ºC refrigerator.

**Preparation of working medium:**
To 80 ml of plain RPMI-1640 medium, 20 ml heat-inactivated fetal calf serum and 1000 U/ml sodium heparin was added. This medium was directly used to set the culture.

**Preparation of reagents:**

**Hypotonic solution (0.075 M):** 0.56 gm potassium chloride was dissolved in 100 ml triple distilled water.

**Carnoy’s Fixative (1:3 Acetomethanol):** 100 ml Carnoy’s fixative was prepared by mixing 25 ml glacial acetic acid (Qualigen, Excel-R) and 75 ml methanol and stored in refrigerator.

**Cytochalasin-B:** 5 mg Cytochalasin-B was dissolved in 5 ml DMSO, sterilized with syringe filter using 0.45 micron nylon membrane and was stored at −20°C in dark till it was used.

**Phytohaemagglutinin:** 5 mg Phytohaemagglutinin-M (PHA-M) was dissolved in 5 ml of sterile triple distilled water, stored at −20°C in dark.

**Giemsa Stain:**

**Sorensen’s Buffer (pH 7.0):** 5.26 gm potassium phosphate monobasic (KH₂PO₄) and 8.65 gm sodium phosphate dibasic (Na₂HPO₄) were dissolved in 1000 ml triple distilled water.

**Stock solution:** 1 gm Giemsa was dissolved in 54 ml glycerol and heated in shaking water bath at 60°C for 2-3 hrs, allowed to cool at room temperature and 84 ml methanol was added. Filtered twice with whatman filter paper no.1 and stored in amber coloured bottle at room temperature.
**Working solution:** 50 ml of 4% working Giemsa stain was prepared by mixing 2 ml of stock Giemsa stain with 24 ml distilled water and 24 ml of Sorensen’s buffer.

**Colchicine:** 1 mg colchicine was dissolved in 10 ml of sterile triple distilled water and stored at 4°C in dark.

### 5.2 Procedure for chromosomal aberrations (CA):

**Culture set-up:**
Peripheral blood lymphocyte culture was set-up according to the standard protocol (Hungerford, 1965) with slight modifications. Peripheral whole blood cultures were set up in duplicate by inoculating 0.5 ml blood in 4.5 ml working culture media and 0.1 ml of phytohaemagglutinin (PHA) added. The cultures were incubated at 37°C in an incubator for 72 hrs. The cultures were gently mixed two times at every 24 hrs to avoid clumping and to stabilize the pH of the media. At 69th hour, 30 μl of colchicine was added to cultures to arrest the cell division at metaphase stage.

**Harvesting:**
The cultures were harvested at 72 hrs i.e. after three hrs of colchicine treatment. The culture tubes were centrifuged at 1000 rpm for 10 min and supernatant medium was discarded. Five milliliters of freshly prepared pre-warmed (37°C) 0.56% KCl hypotonic solution was added. Hypotonic treatment was given for 20 min. The cells were then fixed in chilled Carnoy’s fixative. The cells were thoroughly washed with fixative 3-4 times prior to preparation of the final cell suspension.

**Slide Preparation:**
The slides were kept in distilled water at 4°C in refrigerator. About 3 to 4 drops of the cell suspension was dropped evenly from an angle of 45° on a chilled wet slide from pasteur pipette.
Giemsa Staining:
The slides were dipped in 4% Giemsa stain in Sorensen’s buffer for 5 min. After rinsing in distilled water the slides were scanned under oil immersion lens for at least 100 well spread metaphases and were analyzed for different chromosomal aberrations viz., chromosomal breaks and gaps, chromatid breaks and gaps, acentric fragments and dicentric chromosome using light microscope (Leica, Germany).

5.3 Cytokinesis blocked micronucleus assay (CBMN):
In the CBMN assay, once-divided cells are recognized by their binucleated (BN) appearance after blocking cytokinesis with cytochalasin-B an inhibitor of microfilament ring assembly required for the completion of cytokinesis. Restricting scoring of MN in binucleated cells prevents confounding effects caused by sub optimal or altered cell division kinetics, which is a major variable in micronucleus (MN) assay protocols that do not distinguish between non-dividing cells that cannot express MN and dividing cells that can express. Because of its reliability and good reproducibility, the CBMN assay has become one of the standard cytogenetic tests for genetic toxicity.

Culture Set-up:
Lymphocyte cultures were set up by adding 0.5 ml of whole blood to 4.5 ml of working culture medium. Lymphocytes were stimulated by addition of 0.1 ml phytohaemagglutinin and incubated for 72 hrs at 37°C. A cytochalasin B (Cyt-B) solution was added at a final concentration of 6 µg/ml (French, 1993; Surrallés *et al.*, 1994) to the cultures after 44 hrs incubation to arrest cytokinesis. The cultures were harvested by centrifugation at 1000 rpm for 10 min after 72 hrs of incubation. The cell pellet was treated with a hypotonic solution (1–2 min in 0.075 M KCl at 37°C). Cells were then centrifuged and a Carnoy’s fixative was gently added. Cells were washed twice with fixative and the resulting cells were resuspended in a small volume of fixative solution and dropped onto clean micro slides. Cells were then stained with 10% Giemsa in Sorensen’s buffer, pH 6.8, for 10 min. For each individual at least thousand
binucleates were scanned.

**Scoring method and criteria:**

All slides were scored using a Leica LMBA light microscope at 1000X magnification under oil immersion. Scoring criteria for MN was performed as described by Fenech (1993). And also the scoring criteria adopted by the HUMN project for analysis of micronuclei (Fig. 5) [Fenech et al., 2003]. Only binucleated cells with both nuclei situated in the same cytoplasm were scored. The nuclei were more or less equal in size with intact cytoplasm. The diameter of the micronuclei was not greater than one-third of the main nucleus and had the same staining intensity as the main nucleus. Micronuclei could touch but not overlap the nucleus. The following guidelines for scoring necrotic and apoptotic cells were used:

![Figure 5: The various possible fates of cultured cytokinesis - blocked cells following exposure to cytotoxic/genotoxic agents](Adapted from Fenech et al., 2003).
(i) Cells showing chromatin condensation with intact cytoplasmic and nuclear boundaries as well as cells exhibiting nuclear fragmentation into smaller nuclear bodies within an intact cytoplasm/cytoplasmic membrane were classified as apoptotic;

(ii) Cells exhibiting a pale cytoplasm with numerous vacuoles and damaged cytoplasmic membrane with a fairly intact nucleus as well as cells exhibiting loss of cytoplasm and damaged/irregular nuclear membrane with a partially intact nuclear structure were classified as necrotic.

One thousand cells were counted and scored as either mononucleate, binucleate, trinucleate, tetranucleate, apoptotic or necrotic and ratios for these types of cells were calculated. The numbers of MN in 1000 binucleated (BN) cells were scored and the frequency of MN and nuclear buds and distribution of MN in BN cells were calculated.

5.4 Single cell gel electrophoresis for DNA damage quantification:

Alkaline comet assay: (Singh et al., 1988) with minor modifications

The assay works upon the principle that strand breakage of the supercoiled duplex DNA leads to the reduction of the size of the large molecule and these strands can be stretched out by electrophoresis. Also, under highly alkaline conditions there is denaturation, unwinding of the duplex DNA and expression of alkali labile sites as single strand breaks. Comets are formed as the broken ends of the negatively charged DNA molecule become free to migrate in the electric field towards the anode. Two principles in the formation of the comet are;

1. DNA migration is a function of both size and the number of broken ends of DNA.

2. Tail length increases with damage initially and then reaches a maximum that is dependent on the electrophoreteric conditions, not the size of fragments.
Agarose Slide Preparation:
Normal melting point agarose (1% NMPA) was dissolved in triple distilled water (TDW) by boiling. Eighty microlitres of this solution was smeared on microscopic glass slides as a base coat and air-dried. The blood lymphocytes (10 µl) was mixed with 80 µl of 0.5% low-melting-point agarose (LMPA in PBS) and 100 µl of the cell-agarose mixture was dropped on 1% NMPA (in PBS) precoated slides. Cover slips were placed on the second layer until, it is solidified at 4°C. The cover slip was removed taking care that gel layer remains intact and again 100 µl of 0.5% LMPA (in PBS) was overlaid above the earlier gel layer and solidified as mentioned above.

Lysis and Electrophoresis:
The slides were immersed overnight in a chilled lysis buffer solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, 0.3 M NaOH; pH adjusted to 10 whereas, 1% Tritón X-100, and 10% DMSO, were added just before use). Following cell lysis, slides were placed in a horizontal gel electrophoresis chamber filled with alkaline solution (0.3 M NaOH and 1 mM EDTA, pH>13) allowing it to equilibrate for 20 min. After unwinding, electrophoresis was conducted at 25V, 300 mA for 30 min in a chilled buffer. The slides were drained and neutralized three times by using 0.4 M Tris, pH 7.5, for 5min each. Finally, the treated slides were dehydrated with 80% methanol at room temperature and stored in dark until analyzed.

DNA Staining and Comet Visualization:
The fluorescent dye ethidium bromide (5µg/ml) [Sigma Aldrich, USA] was used and magnification of 400X was used for comet visualization. Slides were scored using an image-analysis system attached to a fluorescence microscope (DMLB, Leica, Germany) equipped with appropriate filters. The microscope was connected to a computer through a charge coupled device (CCD) monochromatead COHU camera to transport images. The measurement was performed using public domain PC-image analysis program CASP (version 1.2.2) software (Fig. 6). Fifty nucleoids (nuclei after lysis) or comets were scored in each sample. The comet parameters
analyzed by CASP were: length of head and length of tail of comet (in pixels), % of DNA in the comet’s head and in the tail, comet length, the tail moment (arbitrary units) and olive tail moment (arbitrary units). The Olive tail moment (OTM) is [percent of DNA in the tail] x [distance between the center of gravity of DNA in the tail and that of center of gravity of DNA in the head in x-direction]. Results are expressed as mean ± SE.

Figure 6: Comet analysis by CASP software

5.5 BUCCAL CYTOME ASSAY (BMCyt)

The BMCyt assay has been used to measure biomarkers of DNA damage (micronuclei and/or nuclear buds), cytokinetic defects (binucleated cells) and proliferative potential (normal cell frequency) and/or cell death (condensed chromatin, karyorrhexis and karyolytic cells) (Fig. 7). The following BMCyt protocol was adopted for buccal cell collection; slide preparation, cellular and
nuclear staining and scoring criteria (Thomas et al., 2009). The various cell types and aberrations that are scored in the BMCyt assay are illustrated below.

Figure 7: The various types of buccal cells following the exposure to cytotoxic/genotoxic agents done on the basis of scheme proposed by Tolbert et al., 1992.

**Cell sampling and preparation:**
Buccal cells were collected from chewers and non-chewers using a modified version of the method used by Belie˘n et al., 1995. Prior to Buccal cell collection the subjects were asked to rinse the mouth thoroughly with tap water to remove any unwanted debris. Wooden spatula was rotated in a circular motion against the inside of the cheek, starting from a central point and gradually increasing in circumference to produce an outward spiral effect. The heads of the spatula were placed into 30 ml PBS at pH 7.0 and agitated.
to dislodge the cells and spun for 10 min at 1500 rpm. Supernatant was removed and replaced with 10 ml of fresh PBS. The PBS helps to inactivate endogenous DNAase and aids in removing bacteria that may complicate scoring. Cells were spun and washed twice, with a final volume of 0.1 ml of PBS being added to the cells. Cells were dispersed on clean micro slides and spread onto the slides. Slides containing cells were air-dried for 10 min and then fixed in ethanol: acetic acid (3:1) for 10 min. Slides were air-dried prior to staining.

**Feulgen staining:**
Fixed slides were treated for 1 min each in 50 and 20% ethanol and then washed for 2 min in deionized water (Milli-Q water purification system). Slides were treated in 5 M hydrochloric acid for 30 min and then washed in running tap water for 3 min. Slides were drained but not allowed to dry out before being treated in Schiff’s reagent in the dark for 90 min at 4ºC. Slides were kept in tap water for 10 min and rinsed well in deionized water for 1 min. Slides were stained for 30 sec in 0.2% light green and rinsed well in deionized water for 2 min. Slides were allowed to air-dry, covered with coverslips and mounted in DPX. Nuclei and MN were stained magenta, while the cytoplasm appears green. The micronuclei were observed at 40X, 63X and 100X magnifications using light microscope (DMLA, Leica, Germany).

**Scoring criteria:**
The various distinct cell populations were determined based on criteria outlined by Tolbert et al., (1991). These criteria are intended to classify buccal cells into categories that distinguish between ‘normal’ cells and cells that are considered ‘abnormal’, based on nuclear morphology. These abnormal nuclear morphologies are thought to be indicative of DNA damage or cell death.

**Normal differentiated cells.** They are distinguished from basal cells by their larger size and by a smaller nuclear to cytoplasmic ratio. No other DNA containing structures apart from the nucleus are observed in these cells.
**Cells with MN.** These cells are characterized by the presence of both a main nucleus and one or more smaller nuclei called MN. The MN are usually round or oval in shape and their diameter may range between 1/3 and 1/16 the diameter of the main nucleus. Cells with MN usually contain only one micronucleus. It is possible but rare to find cells with more than six MN. The nuclei in micronucleated cells may have the morphology of normal cells or that of dying cells (i.e. condensed chromatin cells). The MN must be located within the cytoplasm of the cells.

**Cells with nuclear buds.** These cells have nuclei with an apparent sharp constriction at one end of the nucleus suggestive of a budding process, i.e. elimination of nuclear material by budding. The nuclear bud and the nucleus are usually in very close proximity and are apparently attached to each other. The nuclear bud has the same morphology and staining properties as the nucleus; however, its diameter may range from a half to quarter of that of the main nucleus.

**Binucleated differentiated cells.** These cells have two nuclei instead of one. The nuclei are usually very close to each other and may be touching. The nuclei usually have the same morphology as that observed in normal cells.

**Karyorrhectic cells.** These cells are characterized by the more extensive appearance of nuclear chromatin aggregation (relative to condensed chromatin cells) leading to fragmentation and eventual disintegration of the nucleus. These cells may be undergoing a late stage of apoptosis but this has not been conclusively proven.

**Karyolytic cells.** In these cells, the nucleus is completely depleted of DNA and apparent as a ghost-like image that has no Feulgen staining. These cells thus appear to have no nucleus. It is probable that they represent a very late stage in the cell death process but this has not been conclusively proven.
Scoring method:
One thousand differentiated cells were scored per subject for the various cells types outlined in the buccal cytome assay. These consisted of cells constisted MN, nuclear buds, basal cells, binucleates and the cell death parameters condensed chromatin, karyorrhectic, and karyolytic cells.

5.6 Bacterial artificial chromosome BACs and FISH (LSI p53 & CEP 17)

Stab preparation: The mixture containing LB (Luria Bertani) medium (Bacterial yeast extract 5 gm, Bacto trypton 10 gm, NaCl 10 gm; H₂O up to 1 litre) and 1-1.5% of agar was boiled for 1 min in a microwave. The temperature was monitored until it falls to approximately 45–50°C and the melted agar was poured enough into each sterile plastic petri dish (for stabs).

Stabs: Using a sterile straight wire, a single colony was picked from a freshly streaked plate and it was stabbed deep down into the soft agar several times. The vial was incubated at 37°C for 8–12 hrs leaving the cap slightly loose. The vial tightly was sealed and stored in the dark, preferably at 4°C.

BAC Mini preparation: The inoculation wire was inserted in bacterial stabs [stab (bacteria containing the probe (human DNA), in agar)] (From Dr. Mariano Rocchi / Dr. Nicoletta Archidiacono, http://www.biologia.uniba.it, Italy). Bacteria was grown in 10 ml bacterial medium containing the antibiotic [LB Medium: BAC - Chloramphenicol (12.5 μg/ml); Plasmid artificial chromosome (PAC) - Kanamycin (50 μg/ml)] in 50 ml falcon tubes for 16 hrs.

BAC: For p53 specific for 17p13.1;1). RPII 599B13 2). RPII 89D11

PAC: For CEP 17

PAC-BAC Miniprep for plasmid DNA isolation:
- The medium was centrifuged for 7 min at 4000 rpm.
- The pellet was resuspended completely in 300 µl of GTE (50 mM Glucose, 25 mM Tris, pH 8 and 10 mM EDTA) and the cell suspension was transferred into 2.2 ml of eppendorf tubes.
- About 600 µl of freshly prepared denaturation solution (0.2 N NaOH, 1% SDS) was added and mixed several times by inverting. The lysis was not exceeded for more than 5 min and appeared viscous.
- Further, 500 µl of 7.5 M ammonium acetate was added and mixed immediately by inverting several times. The mixture was left on ice for 10 min and inverted several times during the incubation period and centrifuged at 13000 rpm for 20 min.
- The supernatant was poured into fresh 2.2 ml eppendorf tubes and a second centrifuge at 14000 rpm for 10 min was carried out.
- The supernatant was again poured into fresh 2.2 ml eppendorf tubes.
- Isopropanol (700 µl) was added and mixed several times by inverting.
- The mixture was centrifuged at 14000 rpm for 20 min. The supernatant was discarded and the pellet was barely visible.
- The pellet was washed with 500 µl of 70% ethanol and centrifuged at 14000 rpm for 5 min. The supernatant was discarded.
- The pellet was resuspended in 100 µl of Tris EDTA buffer by taping the tubes.
- It was further treated with RNAse at a final concentration of 100 µg/ml at 37°C for 30 min.
- The DNA was precipitated with 1/10 volumes of sodium acetate and 3 volumes of ethanol. It was incubated at –20°C for 20 min.
- It was centrifuged for 15 min at 14000 rpm and washed with 70% ethanol.
- Resuspended in TE buffer (100 µl) and checked on gel by electrophoresis. Store the DNA at 4°C (Fig. 8a).

**Probe labelling with Biotin by nick-translation:**

**Direct labeling:**

- Band intensity was observed under UV for content of DNA visually.
• Incubate 10 µl (1000 ng) of DNA in 50 µl of Nick translation reaction (Vysis, USA) (Nick enzyme 5 µl, Nuclease free water to adjust volume) in water bath at 15°C for overnight.
• About 5 µl (1/10 volume of nick reaction) of nick product was taken & run on 1.5% agarose gel
• Observed under UV for intensity in comparison with markers to get an idea of presence and concentration of BAC/PAC DNA (Fig. 8b).
• In 5 µl Nick product, 1 µl i.e. 1 ng COT-1 DNA (optional + 1 ng placental or normal human DNA), 1.2 µl of 3 M Na Acetate, pH 8 and 30 µl 100% ethanol was added (in a 1.5 ml Eppendorf tube) and vortexed gently.
• Incubated at 15 min on ice and spinned at 12000 rpm at 30 min in 4°C.
• Supernatant was removed by using pipette and allowed to air dry at RT for 15-30 min.
• Resuspended in 3 µl of triple distilled water and 7 µl hybridisation buffer (Vysis for p53 LSI and CEP-17 CEP) kept in −20°C for FISH.

Figure 8: Identification of product from (a) extracting genomic DNA and (b) Nick products

Principle

Fluorescent in situ hybridization (FISH) identifies or labels, target genomic sequences so their location can be studied. DNA sequences from appropriate,
chromosome specific probes are first labeled with reporter molecules. The labeled DNA probe is then hybridized to the metaphase chromosome or interphase nuclei on a slide. After washing, the specimen is screened for the reporter molecules by fluorescent microscopy.

**Slide preparation and pretreatment**

Slides were treated and denatured essentially as described previously same as chromosomal aberration. Fresh prepared sample slides were pretreated in 2x SSC, 0.5% NP 40, pH 7.0 at 37°C for 15 min and dehydrated in 70%, 85% and 100% ethanol for 1 min each. Air-dried at room temperature.

**In situ hybridization**

The probes used were an α-satellite, biotin-labelled DNA probe that hybridizes specifically with the centromeric region of chromosome 17 and a p53 locus specific probe, directly labelled with Cy3. Slide was denatured for 3 min at 73 ± 1°C. Five microlitres of denatured hybridization mixture (70% Formamide/2x SSC) were immediately pipetted onto the denatured and dehydrated in 70%, 85% and 100% ethanol for 1 min each. Air-dried at room temperature, same time probe was also denatured at 73 ± 1°C for 3 min. About 5 µl of probe was added to slide and covered with coverslip and sealed with rubber cement. Slides were then incubated overnight at 37°C in a moist chamber. The next day, three washes in 2x SSC/0.1% NP 40, for 2 min at RT. Then, 2 min at 72 ± 1°C followed by a wash in 0.4x SSC/0.30% NP 40 and dried with sequential washes of 70%, 90% and absolute ethanol. The nuclear material was counterstained with 0.01 g/ml DAPI in antifading solution. All the slides were stored at 4°C until required for microscopy.

**Microscopic analysis and scoring**

Microscopic analysis was performed on a fluorescence microscope (DMLB, Leica, Germany) equipped with appropriate filters, a 100W mercury lamp and a 1000x magnification objective with iris aperture. A total of 1000 cell was examined for each subject. Cells were classified according to the number of green (17cen) and red (p53 locus) signals.
**Fluorescence in situ hybridization (FISH) Pretreatment Slides**

- Warm metaphase spread to ambient temp.
- Pretreatment fresh prepare sample slides in 2x SSC, 0.5% NP40, pH7.0 at 37°C for 30 min
- Place slides in 70%, 85%, 100% ethanol at room temp./2 min.

**Slides Treatment**

- Prewarm denaturation buffer (70% Formamide/2X SSC, pH 7.0 at 72°C ±1°C) for 3 min.
- Place slides in 70%, 85%, 100% ethanol at room temp./1 min.
- Gently towel dry underside of slide
- Place slide on warmer at 45°C until ethanol evaporation

**Probe Treatment**

- Denature at 73°C for 3 min
- Place on warmer at 45°C & apply probe on denature slide

- Add 5 µl probe to slide, Apply clean 18 x 18 mm coverslips to slide
- Place slide, sealed with rubber cement, humidified chamber chamber
- Incubate at 37 °C for 16 hrs
- Next Day, Remove rubber cement, slide off cover slips
- Wash slide in 1x post buffer II (2x SSC/0.1% NP40) 2 min at RT
- Wash slide in 1x post buffer I (0.4x SSC/0.30% NP40) 2 min, 72°C ±1°C
- Wash slide in 1x wash buffer II for 1 min and Dehydrate in 70%, 85% and 100% ethanol
- Apply 15 µL DAPI/Antifade & place cover slip & Proceed with Microscopy

Air dry at room temperature.
5.7 Cotinine by HPLC

The measurement of cotinine was carried out in representative number of samples at Microbiology laboratory, NIOH, Ahmedabad using HPLC.

Reagents and Standards:
Acetonitrile (HPLC grade from JT Bekars), methanol, glacial acetic acid, dicromethane (DCM), isopropanol (IPA), ammonia (HPLC grade) and sodium acetate (Excelar grade) from Qualigens fine chemical India and HPLC water were used throughout the study. Cotinine drug standards in methanol were obtained from Sigma (USA). All liquids used for experiments were filtered through 0.22 µm membrane filter from Millipore (USA).

Sample Acquisition:
The blood sample was centrifuged at 14,000 rpm for 10 min to separate out the plasma. The plasma was then transferred to a clean tube and stored at -20°C for preservation. Before use, the plasma sample was thawed at room temperature. The remaining sample was placed back into -20°C freezer for storage.

Standards Creation:
Blood plasma samples were equilibrated with a D3–cotinine Internal Standard (ISTD). 100 µL of plasma of various Cotinine concentrations were mixed with 100 µL of ISTD in 2 mL Eppendorf tubes. The following are the cotinine concentrations that were used: 0.0, 100, 200, 500, 600, 700, 800, 900 and 1000 ng/mL. These standards were used to measure assay and machine accuracy and recovery during HPLC analysis.

Sample Protein Precipitation:
500 µL of Acetonitrile was mixed into each tube to precipitate out heavy proteins. The mixtures were vortexed for 2 min and then centrifuged for 10 min at 14,000 rpm in order to form a cell debris pellet. 500 µL of the supernatant was then placed in clean glass test tubes and placed in a N₂ vaporizer for 15 min to evaporate the liquid from the supernatant.
Sample Reconstitution:

Each glass test tube was reconstituted with 400 μL of methanol (MeOH) to dissolve the Cotinine and any remaining proteins. The tubes were again vortexed for 30 sec to further precipitate out any remaining heavy proteins. The solution in each tube was transferred to 400 μL vials for HPLC analysis.

Instrumentation:

Chromatographic analysis was performed on a Shimadzu, Japan LC-10AVP System, consisting of binary gradient pumps, a Rheodyne manual injector with 20 loop, thermostated column oven and PDA detector. The stationary phase was a Lichrospher, RP-18e endcapped (Merck) Lichrocart 250 x 4 mm, 5 μm with guard column the system was monitored by class VP software (version 6.12 SP4).

Assay condition:

Mobile phase consisted of acetate buffer 85% (0.03 M sodium acetate and 0.1 M glacial acetic acid) and acetonitrile (15% (v/v)), containing 0.02 M sodium octanesulfonate as an ion pair agent. The pH of the mobile phase was adjusted to 3.6 with triethylamine to prevent the co-elution of caffeine with cotinine and to minimize the problem of peak tailing. The mobile phase was degassed by filtration under reduced pressure with glass filter assembly using HAWP filter (0.22 μm) from Millipore followed by ultrasonication in transonic digital ultrasonic cleaning bath (ELMA, Germany) for 15 min.

The PDA detector was monitored at 259 nm wavelengths and column was kept at 40°C in column oven. The flow rate was 1ml/min.

Linearity:

Calibration curves were prepared by processing various concentrations, i.e. 100, 200, 500, 600, 700, 800, 900 and 1000 ng/ml of working standard of cotinine prepared by diluting stock standards in mobile phase. An amount of 0.1 ml of each concentration was spiked in mixture of 0.1 ml serum and processed it through conditioned Drug Test-1 column. Eluent was concentrated under nitrogen stream. Twenty micro liters from each concen-
tration was injected into HPLC. The peak areas of cotinine were plotted versus the original concentrations and evaluated by linear least square regression analysis.

Recovery and reproducibility:

The recovery of cotinine was measured under the extraction conditions described above. The recovery was calculated by comparing the peak area ratio of the spiked standards with those of standards injected directly without extraction. Reproducibility of the method was checked by intra- and inter-day variations. Three different concentrations 300 and 400 ng/ml were processed and checked the intra-day variations by injecting five times the same concentrations on the same day, while inter-day variations were checked by running the three standards every day.

Limit of detection (LOD) and limit of quantification (LOQ):

The limit of detection and limit of quantification for cotinine were calculated by the VP software programme provided by Shimadzu. The LOD and LOQ values were calculated by considering signal to noise ratio 3.3 and 10, respectively.

Calibration Curve

Calibration cave was prepared by processing various concentrations i.e. 0.2, 0.4, 0.8, 1.0, 1.2, 1.4 and 1.6 μg/ml of cotinine spiked in plasma of non-tobacco users (Fig. 9). The peak areas were plotted versus the original concentrations and evaluated by linear least square regression analysis using VP software programme. The acceptable value and the correlation coefficient \( r \) should be equal to more than 0.99.

Fig. 10 (a,b,c) shows the chromatogram of direct standard, chromatogram of extracts of blank plasma and chromatogram of blank plasma spiked with cotinine. The total run time was 10 min with cotinine eluting at 6.3 min. There is no peak in the extract of non-chewers/non-smokers at the time of elution of cotinine.
Figure 9: Calibration curve of spiked standards of cotinine

a). Chromatogram of Cotinine Direct Standard- 0.6 µg/ml
b). Chromatogram of Cotinine Spiked Standard- 0.6 µg/ml

c) Chromatogram of blank plasma

Figure 10: Chromatograms of spiked standards
Recovery and reproducibility:

The recovery of cotinine was measured under the extraction conditions. The recovery for the cotinine was in the range of $74.64 \pm 1.96$ to $83.18 \pm 2.77$ (Mean ± SD) with the average value of $80.49 \pm 2.77$ (Mean ± SD). The reproducibility of the method was demonstrated by repeated injections of plasma spiked with cotinine standard.

Limit of detection and limit of quantification:

The limit of detection was 5 ng/ml and limit of quantification values was 10 ng/ml for cotinine, as calculated by signal to noise ratio with the help of VP software programme provided by Shimadzu Ltd., Japan.

5.8 Zinc and Copper by flame atomic absorption spectrometry

Atomic Absorption Spectrophotometry is designed to determine the amount (concentration) of an object element in a sample, utilizing the phenomenon that the atoms in the ground state absorb the light of characteristic wavelength passing through an atomic vapor layer of the element.

Basic Principle:

The technique of flame atomic absorption spectroscopy (FAAS) requires a liquid sample to be aspirated, aerosolized, and mixed with combustible gases, such as acetylene and air or acetylene and nitrous oxide. The mixture is ignited in a flame whose temperature ranges from 2100 to 2800°C. During combustion, atoms of the element of interest in the sample are reduced to free, unexcited ground state atoms, which absorb light at characteristic wavelengths. The characteristic wavelengths are element specific and accurate to 0.01-0.1 nm. To provide element specific wavelengths, a light beam from a lamp whose cathode is made of the element being determined is passed through the flame. A device such as photon multiplier can detect the amount of reduction of the light intensity due to absorption by the analyte, and this can be directly related to the amount of the element in the sample. The
diagrammatic representation of principle of the analysis of metal is shown in Fig. 11.

Figure 11: The theory and instrumentation of the atomic absorption spectrophotometer

Pretreatment or dilution of serum:

The serum samples were brought to the room temperature and diluted with deionised water for metal estimation as follows:

For the determination of copper and zinc in serum, the sample was diluted with an equal volume of deionised water (1:3). The same dilution was used throughout the study.

Analytical Procedure for estimation:

Diluted serum samples were brought to trace metal laboratory, Hygiene Department, NIOH, for copper and zinc analysis. These samples were analyzed using Atomic Absorption Spectrophotometer (AAS), Perkin Elmer Double beam Model No.3100, USA.

The lamp and wavelength used for analysis of Cu and Zn are as follows:
Lamp used for Cu: Hollow Cathode Lamp, Lumina.
Wavelength for Cu: 324.7 nm.
Lamp used for Zn: Electroless Discharge Lamp.
Wavelength for Zn: 213.9 nm.

The standards used for the above analysis were of E Merck (ICP Grade).

The sample readings were taken and multiplied with appropriate correction factor.

**Calculation:**

A standard curve was plotted considering the absorbance of standards. The absorbance of copper and zinc in the sample was plotted on standard curve and the concentration of zinc and copper in the sample was obtained after multiplying with the dilution factor.

**6. STATISTICAL METHODS:**

All statistical analyses were conducted using either SPSS 16.0 for windows (SPSS, Chicago, Illinois, USA). Descriptive statistics are reported as means and SE for normally distributed measures. Student’s unpaired ‘t’ test was performed to compare variables between chewers and non-chewers. Analysis of variance (ANOVA) was performed using tukey’s test for the analysis of chewers, non-chewers and OSMF subjects and also among within chewers group. Normality was ascertained using the Shapiro–Wilk test and visual examination of histograms. Univariate analysis of variance (ANOVA) was used to identify significant differences in means among study groups. Differences in these groups subsequently tested by using either Hochberg’s GT2 or Tamhane’s T2 post-hoc test, based on the results of Levene’s Test for equality of variance. Correlation between Lifetime chewing exposure (LCE) and cytogenetic parameters was evaluated by using the Pearson’s correlation coefficient and linear regression analysis. For two group comparison independent samples t-test was used however for the entire statistical test significance level is $P<0.05$. 
