MATERIALS AND METHODS

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The present study was planned with the central objective of assessing the role of antioxidants in health and diseases during aging process. The related objectives were to study the information on local adults and elderly population with respect to socio demography, diet, nutritional and lifestyle related factors. The study also included collecting similar data on adults and elderly with cardiovascular diseases (CVD) and oral cancer. The other objectives also included assessment of antioxidants in diet as well as blood in above mentioned subjects. Further, a small sample of care givers was also studied for knowledge and practices (KaP) with respect to health benefits of antioxidants rich foods for elderly. The healthy adults and elderly subjects for this study were selected from the free living population of Vadodara city. Subjects with CVD were selected from Jivraj Mehta Hospital, Ahmedabad whereas the subjects with oral cancer were selected from SSG Hospital, Vadodara. The caregivers of the elderly were also selected from Vadodara city. The details of the plan of work, tools and techniques used in the study are described below:

PLAN OF STUDY

For collecting detailed information on various aspects concerning socio-demography, nutrition, diet, activity, addiction and disease profile of the subjects, the study was divided into four sections as follows :

I. EVALUATION OF SOCIO-DEMOGRAPHIC PROFILE, NUTRITIONAL STATUS, DIETARY PATTERN, LIFESTYLE FACTORS AND ANTIOXIDANTS PROFILE IN FREELIVING SUBJECTS (AGED 45 YEARS AND ABOVE).

II. ASSESSMENT OF SOCIO- DEMOGRAPHIC PROFILE, NUTRITIONAL STATUS, DIETARY PATTERN, LIFESTYLE FACTORS, CLINICAL AND ANTIOXIDANTS PROFILE IN SUBJECTS SUFFERING FROM CARDIO VASCULAR DISEASES (45 YEARS AND ABOVE).

III. COLLECTION OF DATA ON SOCIO- DEMOGRAPHIC PROFILE, NUTRITIONAL STATUS, DIETARY PATTERN, LIFESTYLE FACTORS, CLINICAL AND ANTIOXIDANTS PROFILE AND ORAL HYGIENIC PRACTICES IN SUBJECTS WITH ORAL CANCER (45 YEARS AND ABOVE).

IV. ASSESSMENT OF KNOWLEDGE AND PRACTICES OF THE CAREGIVERS REGARDING ROLE OF ANTIOXIDANTS IN THE DIET OF THE ELDERLY.

SECTION I

EVALUATION OF SOCIO-DEMOGRAPHIC PROFILE, NUTRITIONAL STATUS, DIETARY PATTERN, LIFESTYLE FACTORS AND ANTIOXIDANTS PROFILE IN FREE-LIVING SUBJECTS (AGED 45 YEARS AND ABOVE).

This section dealt with collection of the baseline data of the subjects from the free living population with respect to socio-demographic attributes, nutritional status, dietary intakes, activity pattern, addiction pattern and disease profile.

Selection of the subjects

Healthy subjects (45 - 59 years) were selected by snow ball technique from the free living population of urban Vadodara. The elderly subjects aged 60 years and above were selected from the senior citizens councils of Vadodara. The criteria for the selection of these subjects were :

a) should not have any chronic diseases
b) should be willingness to co-operate.

SECTION II

ASSESSMENT OF SOCIO-DEMOGRAPHIC PROFILE, NUTRITIONAL STATUS, DIETARY PATTERN, LIFESTYLE FACTORS, CLINICAL AND ANTIOXIDANTS PROFILE IN SUBJECTS SUFFERING FROM CARDIO VASCULAR DISEASES (45 YEARS AND ABOVE).

This dealt with the collection of the baseline data of the patients suffering from Cardiovascular diseases (CVD) with respect to their socio-demography, nutritional status, dietary intakes, activity pattern, addiction pattern and disease profile along with their controls.

Selection of the cases

Subjects suffering from CVD were selected from the Jivraj Mehta Hospital, Ahmedabad. Following criteria was used for the selection:

a) they should be freshly diagnosed confirmed cases of CVDs by the physician
b) should be willing to co-operate.

Selection of the controls

The control group for this section included subjects matched with cases

a) with respect to age and economic status.

b) should not have any chronic diseases

c) should be willing to co-operate.

Biochemical assessment

A sub-sample of the CVD cases and controls were studied for the following :

a. Antioxidants status

1. serum α-tocopherol
2. plasma ascorbic acid
3. serum β-carotene

b. Lipid profile.

1. Total Cholesterol
2. Total Triglycerides
3. HDL-C
4. LDL-C
5. VLDL-C

SECTION III

COLLECTION OF DATA ON SOCIO-DEMOGRAPHIC PROFILE, NUTRITIONAL STATUS, DIETARY PATTERN, LIFESTYLE FACTORS, CLINICAL AND ANTIOXIDANTS PROFILE AND ORAL HYGIENIC PRACTICES IN SUBJECTS WITH ORAL CANCER (45 YEARS AND ABOVE).

This aspect dealt with the collection of the baseline data of the patients suffering from oral cancer along with their controls. The information was collected with respect to the socio-demography, nutritional status, dietary intakes, activity pattern, addiction pattern , oral hygienic practices and disease profile.

Selection of the cases

Subjects suffering from oral cancer were selected from the radiation therapy out patients department of Shri Sayajirao General Hospital, Vadodara. The criteria for the selection were:

a) they were newly diagnosed confirmed cases of oral cancer by a physician

b) should be willing to co-operate.

Selection of the controls

The controls for this section were selected from the free living population of Vadodara. The criteria for the selection of the controls were:

a) should be matched with cases with respect to age and economic status

b) should not have any chronic diseases

c) should be willing to co-operate.

Biochemical assessment

A sub-sample of oral cancer cases and controls were studied to assess their antioxidants status (serum α-tocopherol, plasma ascorbic acid and serum β-carotene) along with the lipid profile and IgM.

SECTION IV

ASSESSMENT OF KNOWLEDGE AND PRACTICES OF THE CARE GIVERS REGARDING THE ROLE OF ANTIOXIDANTS IN THE DIET OF THE ELDERLY.

This section dealt with the assessment of knowledge and practices of the care givers of the elderly with respect to the common health problems of the elderly, protective role of antioxidants rich foods.

Selection of the subjects

The subjects were selected from the free living population of urban Vadodara. The criteria of the selection were :

a) they were care givers to the healthy elderly people

b) they should be willing to co-operate.

Tool and methods used for achieving valid and relevant information regarding various aspects of the study are discussed below.

TOOLS

The tools used for this purpose were:

1) Interview cum questionnaire
2) Set of standard cups and spoons
3) Bathroom scale
4) Measuring Tape

Prior to the actual commencement of the study, testing of all the tools was carried out on a few subjects. The purpose of this test was to check the data gathering procedure and reliability of the measurement. The tools and methodology have been described in brief below.

QUESTIONNAIRE

A carefully planned questionnaire was used to elicit information regarding socio-demographic profile, nutritional status, dietary intake, activity pattern, addiction pattern and health disorders. A questionnaire was also used to collect information on the knowledge and practices of the care-givers of the elder with respect to health benefits of consumption of antioxidants rich foods (Appendix I).

SOCIO-DEMOGRAPHIC PROFILE

This included information on age, education, family income as well as per capita income and ethnic group.

NUTRITIONAL STATUS

The data regarding the nutritional status was collected using anthropometric measurements.

Anthropometry Measurements

The anthropometric measurements of the subjects were assessed with respect to height, weight and body mass index (BMI).

a) Height

Height was measured by using a measuring tape. The subjects were made to stand barefoot, with the back of their head, shoulders, buttocks and heels touching the wall. The head was kept comfortably erect and hands were left hanging loosely by the side. The readings were recorded to the nearest 1 cm. Two readings were taken for every subject.

b) Weight

Bathroom weighing scale was used to measure the weight of all the subjects. The weight was measured with the subjects in their usual clothing, but without shoes. The measurements were made to the nearest 0.5 kg. Two consecutive readings were taken for every subject and the scale was adjusted to zero after each measurement.

c) Body Mass Index (BMI)

The BMI of the subjects were calculated by the following formula :

$$BMI = \frac{\text{Weight (kg)}}{\text{Height } (M^2)}$$

DIETARY PATTERN

The data for the dietary intake was collected using information related to changes made in food consumption along with the 24 hour dietary recall and food frequency.

a) Changes in food consumption

Self reported changes in food consumption, by the subjects in the past 5-10 years was noted. These changes were in terms of foods totally omitted, reduced or increased. The reasons for the change were also noted. This data was collected using a list of food groups that also included items such as fried foods, sweets, sugar, salt.

b) 24 Hour dietary recall

The subjects' dietary intake of one normal day was noted from which their nutrient intake per day was calculated in terms of energy, protein, fat, fiber, calcium, iron, β-Carotene, and Vitamin C. A set of standard cups and spoons were used to make exact measurements of food taken by the subjects while collecting data on food intake per day.

c) Food frequency

The food frequency questionnaire included an exhaustive list of food items rich in dietary as well as non-nutrients anti-oxidants rich foods along with fats.

The food frequency list of foods rich in anti-oxidants included vegetables both green leafy and yellow vegetables both rich in vitamin A and β-carotene. Data on consumption of fruits rich in

vitamin C and β-carotene was also collected using the food frequency list. The list also included list of vegetables rich in isoflavonoids

The food frequency list also included fat rich items like milk and milk products, nuts and oil seeds, non-vegetarian food items, high fat, moderate fat and low fat sweets, shallow fried and deep fried foods. The questionnaire also included a list of food items, which require use of additional fat as topping.

The consumption of these food items was noted along with edible oil consumption in terms of frequency i.e. daily, once a week, twice a week, fortnightly, monthly, rarely or never.

ACTIVITY PATTERN

The 24 hour activity pattern of the subjects was noted and then categorized into heavy, moderate or sedentary activity

ADDICTION PATTERN

Addiction pattern of the subjects was noted with respect to having addiction to pan, ghutka, supari, smoking bidi, cigarette, alcohol consumption and tobacco chewing. The addiction was assessed in terms of number of years of addiction, frequency per day and whether the addiction was quit along with reason.

ORAL HYGIENE

Oral hygiene was assessed in subjects with oral cancer along with their controls in terms of oral care practices.

DISEASE PROFILE

This part of the questionnaire dealt with the disease profile of the subjects and included free listing of the ailments, disease present in the subjects along with antecedent health record for the past 10 years. It also included their major/minor illness, hospitalization, operations, or accidents and allergy (Appendix I). Different types of disorders which could be possible faced by the subject were grouped and listed under the following headings:

1. *Problems of gastro intestinal system*
2. *Problems of respiratory system*
3. *Problems of cardio vascular system*
4. *Problems of genito-urinary system*
5. *Problems of nervous system*
6. *Problems of endocrine system*
7. *Other miscellaneous problems*

KNOWLEDGE AND PRACTICES

An semi structured questionnaire was used to elicit information on the knowledge and practices of the care-givers with respect to physiological changes taking place in the body with advancing age, importance of protective foods in the diets and role of fruits and vegetables in healthy aging (Appendix II).

BIOCHEMICAL ASSESSMENT

Biochemical assessment in terms of antioxidants profile (plasma ascorbic acid, serum α-tocopherol and serum β-carotene) and lipid profile was done on the sub-sample of subjects selected in section II and III. Venous blood samples were collected by the trained technician and following estimations were carried out.

a) Antioxidants status

The antioxidants status was assessed with respect to serum β-carotene, serum tocopherol and plasma ascorbic acid.

(i) SERUM β-CAROTENE

The method for serum β-carotene was given by Dugan et al (1964)

Principle : Proteins are precipitated with absolute alcohol and the β-carotene extracted into light petroleum. The intensity of the yellow colour due to presence of β-carotene is read at 450 nm using a spectrophotometer. The standard β-carotene was obtained from Roche Products Ltd, Switzerland.

Procedure : Pipette 1.0 ml serum into a 15 ml glass - stoppered centrifuge tube, add 2.0 ml ethanol, stopper and mix well with a vortex mixer. Add 3.0 ml light petroleum and place the stoppered tubes in a mechanical shaker for 10 min to extract the retinol and carotenes into the

petroleum section. Centrifuge the tubes for 10 min at 2500 g. Carefully transfer 2.0 ml of the upper, light petroleum section path length, and read the absorbance (A_{450}) at 450 nm against a light petroleum blank.

Calculation : Serum β-carotene (mg/l) = $\frac{A_{450} \text{ of unknown}}{A_{450} \text{ of standard}} \times \text{concentration of standard} \times 10$

(II) SERUM α-TOCOPHEROL

The method for serum α- tocopherol was given by Frank and Baker (1968)

Principle : Serum tocopherols can be measured by their reduction of ferric to ferrous ions which then form a red complex with α,α'- dipyridl. Tocopherols and carotenes are first extracted into xylene and the absorbance is read at 460 nm to measure the carotenes. A correction for the carotenes is made after adding ferric chloride and reading at 520 nm using a sphectrophotometer. The standard tocopherol was obtained from Sigma Chemicals.

Procedure : Into three stoppered centrifuge tubes, measure 1.5 ml serum, standard or water (blank) respectively. To the test and blank add 1.5 ml ethanol and to the standard 1.5 ml water. Add 1.5 ml xylene to each tube, stopper, mix well and centrifuge. Transfer 1.0 ml of each xylene layer into a clean stoppered tube, carefully excluding any protein or ethanol. Add 1.0 ml dipyridyl reagent to each tube, stopper and mix. Pipette 1.5 ml of the mixture into sphectrophotometer cuvettes and read the absorbance (A_{460}) of the test and standard against the blank at 460 nm. Then, in turn, beginning with the blank add 0.33 ml ferric chloride solution, mix, set the wavelength to 520 nm and 1.5 min after mixing read the absorbance (A_{520}) of the test and standard against the blank.

Calculation : Serum tocopherol (mg/l) = $\frac{A' \text{ of unknown}}{A' \text{ of standard}} \times 10$

Where $A' = A_{520} - 0.29 \times A_{460}$

(III) PLASMA ASCORBIC ACID

The method for plasma ascorbic acid was given by Roe (1961)

Principle : The ascorbic acid is converted to dehydroascorbic acid by shaking with acid washed norit and this is then coupled with 2,4 - dinitrophenylhydrazine in presence of thiourea as a mild reducing agent. Sulphuric acid then converts the dinitrophenylhydrazone into a red compound which is assayed at 520 nm using a spectrophotometer. The standard ascorbic acid was obtained from Sigma Chemicals.

Procedure : To 6 ml trichloroacetic acid in a centrifuge tube add 2 ml plasma slowly, with constant stirring to produce a fine suspension. Stand 5 min, centrifuge, then add 300 mg Norit to the supernatent fluid, shake vigorously and filter. Place 2 ml of the filtrate into each of two test tubes. Keep one for the blank and to the other, the test, add 0.5 ml 2,4 - dinitrophenylhydrazine reagent. Stopper and place in a water bath at 37 ° C for exactly 3 h. Remove and place both test and blank in ice-cold water and slowly add 2.5 ml sulphuric acid, drop by drop, taking about half a minute to do so, so that there is no appreciable rise in temperature. Finally add 0.5 ml dinitrophenylhydrazine to the blank. Mix well the contents of both tubes while still in iced water. Remove, and after 30 min read at 540 nm, or using a yellow green filter against the blank. As standard, treat 2 ml of the working standard in the same way as the test.

Calculation : Plasma ascorbic acid (mg/l) = $\frac{\text{Reading of the unknown}}{\text{Reading of standard}} \times 10$

b) Lipid Profile

The parameters estimated for lipids were triglycerides, total cholesterol, HDL - cholesterol, LDL - cholesterol and VLDL.

(I) TRIGLYCERIDES

The TG were estimated by GPO/PAP method using enzymatic kit (Me Gowan, 1983).

Principle : The TG is hydrolysed by lipase to glycerol and free fatty acids. Glycerol is phosphorylated by ATP in the presence of glycerol kinase (GK) to glycerol-3-phosphate (G-3-P-O) producing hydrogen peroxide which reacts with 4-amino antipyrine and 3,5-dichloro 2-hydroxyl benzene sulphonic acid (DHBS) in the presence of enzyme peroxidase

(POD) to produce red quinoeimine dye. The intensity of the colour develop is proportional to the TG concentration in the sample.

Reaction **:**

$$TG + H_2O \xrightarrow{\text{Lipoprotein Lipase}} \text{Glycerol} + \text{Fatty acid}$$

$$\text{Glycerol} + ATP \xrightarrow{GK} \text{Glycerol - 3 - P} + ADP$$

$$\text{Glycerol - 3 - Phosphate} + O_2 \xrightarrow{GPO} \text{Dihydroxy acetone phosphate} + H_2O_2$$

$$H_2O_2 + \text{aminoantipyrine} + DHBS \xrightarrow{POD} \text{Quinoneimine} + H_2O_2$$

(II) TOTAL CHOLESTEROL

TC was estimated by CHOD - PAP method using the enzymatic kit provided by Human (Flegg, 1973).

Principle : Cholesterol esters are hydrolysed by cholesterl esterase (CE) to free cholesterol and fatty acids. Free cholesterol is oxidised by cholesterol oxidase (CO) to cholest - 4 - en - 3 - one and hydrogen peroxide. Hydrogen peroxide produced, couples with 4 - aminoantipyrene and phenol in the presence of peroxidase to form a pink coloured quinoneimine dye. The intensity of colour is proportional to the cholesterol concentration.

Reaction :

$$\text{Cholesterol ester} + H_2O \xrightarrow{CE} \text{Cholesterol} + \text{fatty acids}$$

$$\text{Cholesterol} + O_2 \xrightarrow{CO} \text{cholest - 4 - en - 3one} + H_2O_2$$

$$2\,H_2O_2 + 4 \text{ amino antipyrene} + \text{phenol} \xrightarrow{POD} \text{Quinoeimie dye} + 4\,H_2O.$$

(III) HIGH DENSITY LIPOPROTEIN - CHOLESTEROL

The VLDL and LDL fractions of serum sample were precipitated using phosphotungstic acid (Warnick et al, 1982). The supernatent obtained by the enzymatic kit for TC estimation was used for HDL-C.

(IV) VERY LOW DENSITY LIPOPROTEIN - CHOLESTEROL

The VLDL - C was estimated using the following calculation :

$$VLDL - C = TG / 5$$

(v) LOW DENSITY LIPOPROTEIN - CHOLESTEROL

The LDL - C was estimated using the following calculation :

$$LDL - C = TC - (HDL + VLDL)$$

C) IgM

Immunoturbidimetric assay was used for IgM

Principle : Sample containing human IgM is suitably diluted and then reacted with specific antiserum to form a precipitate which is measured turbidimetrically at 340 nm. By constructing a standard curve from the absorbances of standards, the light concentration of sample is determined.

STATISTICAL ANALYSIS

Database was created in MS-Excel 2000. Statistical analysis was performed using SPSS (version 10.0) and Epi 6 software package.

Socio-demographic attributes : Percent responses were calculated for the variables of the socio-demographic attributes.

Anthropometric parameters : Mean and standard deviation was calculated for height, weight, MUAC, B.M.I of the subjects.

Dietary recall : Mean and standard deviation were calculated for all the nutrients received from the 24 hour dietary recall. Students 't' test was used to compare the means of the nutrients intake by the cases and the controls. Each nutrient intake was compared to the percent of the recommended dietary allowances (RDA) met daily.

Odd's ratio was calculated from the food frequency data to find the association between the consumption of food rich in anti oxidants and fats with the occurrence of cardiovascular diseases and oral cancer. This association was further supported by the Confidence Interval at 95% level. Chi square was also used to see the trend of association between the risk and protective factors with the occurrence of both the diseases selected for the study.

Activity pattern : Percentage of the subjects reporting total time spent in work related, leisure time activities along with sleep were noted..

Biochemical parameters : Significance difference in the biochemical parameters in terms of haemoglobin, antioxidants status (serum β-carotene, plasma ascorbic acid, and serum tocopherol) and lipid profile between the cases and the controls were found out using student's t - test at various levels of significance.

Addiction pattern : Odd's ratio was used to find out the association between the risk of exposure to various addictions with the occurrence of the diseases.

Disease profile : Percent subjects suffering from various diseases were calculated.

Oral hygiene : Percent responses were used to find out the total subjects following proper oral care practices.

Knowledge and practices : Percentage of the care-givers giving acceptable responses to the questions related to the knowledge and practices were calculated.

STRAITIFIED ANALYSIS

Straitified analysis was used to further investigate the risk of developing the cardiovascular disease and oral cancer after adjusting for age. For this purpose, the subjects were divided into two categories viz 45 - 59 years and 60 years and above.

FIGURE 6: EXPERIMENTAL PLAN

SECTION I: COLLECTION OF BASELINE DATA FROM FREELIVING POPULATION (45 YRS AND ABOVE)
(Men=166 and women =170)

Socio-demography	Nutritional status	Dietary intake	Activity pattern	Addiction patterns	Disease profile
* questionnaire	*anthropometry (height, weight, bmi)	* 24 hour dietary recall * food frequency	* self reported	* questionnaire	* Check list

SECTION II: COLLECTION OF DATA ON CVD PATIENTS

CASES
(Men = 63, Women = 38)

CONTROLS
(Men = 63, Women = 38)

Socio-demography	Nutritional status	Dietary intake	Activity pattern	Addiction patterns	Disease profile
* questionnaire	*anthropometry (height, weight, bmi)	* 24 hour dietary recall * food frequency	* self reported	* questionnaire	* Check list

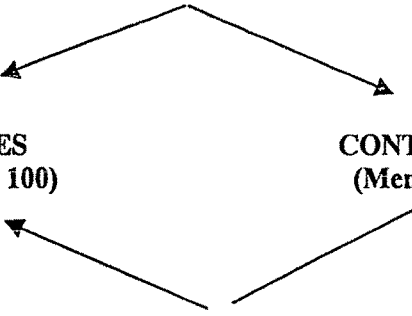
Sub-sample

CASES
(Men=30, women=30)

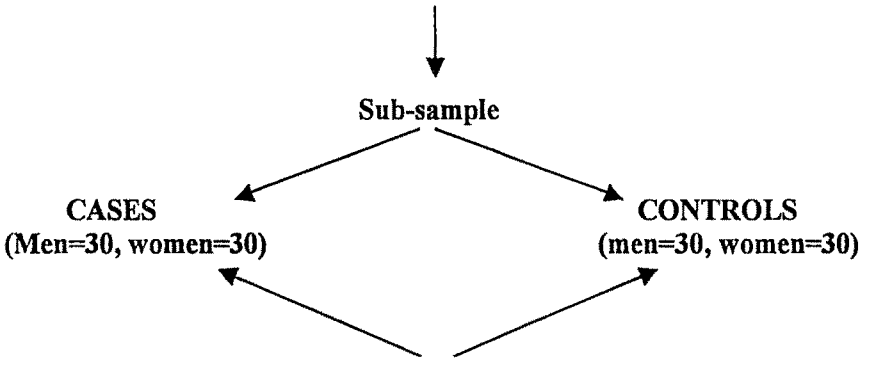
CONTROLS
(men=30, women=30)

- **S.β-carotene (Dugan et al, 1943)**
- **P.vitamin c (Roe & Kuther,1961)**
- **S.tocopherol (Baker & Frank,1968)**
- **Lipid profile (Enzymatic kit)**

SECTION II: COLLECTION OF DATA ON ORAL CANCER PATIENTS



Socio-demography	Nutritional status	Dietary intake	Activity pattern	Addiction patterns	Disease profile	Oral hygiene
* questionnaire	*anthropometry (height, weight, BMI)	* 24 hour dietary recall * food requency	* self reported	* questionnaire	* Check list	*question naire



- S.β-carotene (Dugan et al, 1943)
- P.vitamin c (Roe & Kuther,1961)
- S.tocopherol (Baker & Frank,1968)
- Lipid profile (Enzymatic kit)
- IgM (Kit)

SECTION IV :

TO ASSESS THE KAP OF THE CAREGIVERS WITH RESPECT TO THE ANTIOXIDANTS FOODS AND OVER HEALTH CARE OF THE ELDERLY.