MATERIALS AND METHODS

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As indicated earlier in the introduction the present study was carried out in Trivandrum (Kerala) because of the unique nature of the diet in that, the Kerala diet is relatively poor in magnesium as the water is soft and the staples namely rice and tapioca are both poor in magnesium content. Also the fat used for cooking is coconut oil which is rich in saturated fats and poor in tocoferol. The consumption of such a diet will be low in low income group (LIG) and may create deficiencies while it may be adequate or even more in the high income group (HIG) creating difficulties of over nutrition. Thus the main objectives of this study are -

1. To monitor the pattern of lipid changes with progress of pregnancy and after parturition, to find out to what extent this pattern was affected by the plane of nutrition (LIG and HIG) and

2. To study the aging changes in adult men and women for serum lipids, magnesium and blood glucose. Somatic measurements were also collected as a measure of health and nutritional status in this group.

Selection of subjects:

PART I: THE SUBJECTS SELECTED WERE FROM SRIAVITTAM THIRUNAL HOSPITAL The pregnant subjects used for studies up to 30 week

of pregnancy were those women who visited the antenatal clinics as outpatients. Some of the women with less than 16 week of gestation were also those from the medical termination of pregnancy (MTP) unit. Hence those women who were willing to give blood only could be taken and the number was smaller. However women greater than 30 weeks of pregnancy were those that were admitted in the prenatal ward for delivary at Sri Avitham Thirunal Hospital, Trivandrum. Thus more number of women could be enrolled for the study.

The income levels and occupations were recorded and the subjects were classified as belonging to low or high income groups (LIG and HIG). Those in the former belonged to families of unskilled or semi-skilled workers and had monthly per capita income of less than Rs. 120 - 150. Those in the later belonged to the families of executives, professionals and business groups and had monthly per capita income of Rs. 300/- and more.

The women admitted for delivary were investigated before and most of them after delivary as well, for the different parameters. Also their infants birth weights and blood sample before 36 hours after birth for serum cholesterol were taken. The gestation period of these women was determined by taking menstrual age (as reported by the mother after careful questioning) minus two weeks to allow for

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ovulation. The reliability of the method was determined in this laboratory by comparing the results of prospective and retrospective investigations on a selected sample (Nikita Shah and Rajalakshmi, unpublished).

PART II:

For studies on age related changes, subjects in the age group of 20-70 years of both sexes belonging to high and low income groups were investigated. The subjects were in apparently normal health with no clinical symptoms such as chest pain, palpitation etc.

In summary the following data were collected in these investigations:

PART I

Pregnant and parturient women:

1. Family income and occupation of bread winners.
2. (a) Period of gestation;
   (b) Food consumption.
3. Dietary pattern and intakes of the family by using oral questionnaire method.
4. Body weights and parity.
5. Biochemical examination for
   (a) blood hemoglobin

(b) Serum lipids, cholesterol, phospholipids and triglyceride

(c) Serum magnesium and vitamin E

PART II:

Adult men and women of different ages:

1. Dietary pattern
2. Somatic measurements;
   Height, weight and circumferance of chest, mid-arm and abdomen.
3. Biochemical parameters:
   (a) Blood glucose
   (b) Serum lipids-cholesterol, phospholipids and triglyceride
   (c) Serum magnesium.

An oral questionnaire method was used to get the general information on the family with special reference to dietary pattern, food consumption and family composition. This data was collected at random from willing participants in the two groups.

Diet Survey:

The number of subjects investigated for the study are 153 pregnant women and 357 adult men and women of different ages in the Low and High income groups totalling to 488 subjects in all. To know the dietary and consumption pattern of the people of Kerala dietary composition was calculated from questionnaires on a random sample of 241 subjects (families) (Questionnaire in Appendix).

Subjects for other parameters:

The following tables show the overall number of subjects investigated for the study i.e. study on pregnancy (Table 4a and b) and on aging (Table 6). However, the number of subjects for each parameter studied may vary slightly due to either non co-operation of the subjects at times or due to insufficiency of the sample for the different parameters investigated. Hence the numbers actually investigated for the different parameters are again given in each table for reference.

Somatic measurements:

These were made according to the procedures suggested by Jelliffe (1966) and Buckler (1979).

Table 5: Study on Pregnancy and post-partum:

(a) Mothers:

: Subjects investigated in the High and Low income groups during different gestation periods

Groups	Number of subjects in each gestation period (wks)						Total sample
	Non preg; @ Non lactating (NPNL)	⩽10 and 10 wks	10-20 wks	20-30	30-40	Post-partum*	
HIG	32	13	20	14	27	23	129
LIG	32	13	19	21	26	17	128
		26	39	35	53		257

HIG - High Income Group.
LIG - Low Income Group.

* Subjects in postpartum are also investigated prepartum and are included in the previous group.

@ NPNL - Age and weight matched controls taken from the Aging study.

(b) Infants: Infants included from HIG and LIG as per birth status for serum cholesterol

Groups	Full Term		Prematures
	AGA	SFA	
HIG	10	-	-
LIG	33	12	22

Table 16: Studies on adult amen and women at different ages.

: Adult men and women investigated in the High and Low income groups during different ages.

Age group (Yrs)	High Income Group (HIG)				Low Income Group (LIG)		All	
	Men		Women		Men	Women	Men	Women
	Veg.	Non-veg.	Veg.	Non-veg.				
20 - 29	10	24	7	21	11	24	45	52
30 - 49	18	39	15	32	17	29	74	76
50 - 70 and above	17	22	17	18	17	19	56	54
Total	45	85	39	71	45	72	175	182

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(a) Height : Exact body length was measured with the subject's heels, buttocks and upper back in contact with an upright wall with head erect (The height was marked on the wall) by placing a flat foot rule in a horizontal position at the highest point on the head. The height of the mark was measured in centimeters with an accurately calibrated measuring tape. Heights were measured (with due care regarding its use) to the nearest millimeter.

(b) Weight : Weight was taken to nearest 100g in the fasting condition without footwear and with normal light clothing. The subjects were made to stand comfortably and steadily on a weighing machine (Bath-room scale). The bath-room scales used was checked periodically for reliability with standard grocer's weights. No allowance was made for clothing worn by the subjects as the weight of clothes worn is usually less than 0.4 to 0.6 kg in case of women and men.

(c) Chest circumferance : The subject was made to stand in position, then measured for the growth of the thorax at the levels of the Xiphistermum and in a plane at right angles to the vertibral column below the inferior angles of the scapulae. Only enough tension was applied to enable

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the tape to rest against the parameter of the thorax without slipping. Two measurements both in the position of normal expiration and inspiration were recorded in centimeters and the mean taken as the chest measurement (ICMR Technical Report series, 1972).

(d) Mid-arm circumference : The mid-arm circumference was measured to the nearest 0.1 cm with a flexible steel tape which was placed gently but firmly around the limb to avoid compression of the soft tissue.

The left arm circumference was measured while hanging freely at the mid-point between shoulder and elbow which was selected in the same way as for triceps skinfold.

(e) Abdominal circumference : The abdominal circumference was measured to the nearest 0.1 cm with a flexible tape. The measurement of abdominal circumference was made by passing the tape round the waist line just below the navel area when the subject was standing straight but at ease.

BIOCHEMICAL ESTIMATIONS:

Collection of blood sample:

Intravenous blood samples were collected on all adults in the fasting state early morning in both oxalated and plain bulbs. The samples of blood were brought to the laboratory within one hour of collection. Oxalated blood samples were taken immediately for the estimation of hemoglobin. Serum was collected from the non-oxalated blood samples in stoppered tubes, stored in the refrigerator and analysed within 72 hours of the collection.

From infants - Infant blood samples were collected in the morning at about 7.00 a.m. from the ante-cubital vein within 36 hours after birth. About 0.5 to 0.75 ml of blood was collected and serum was separated. Serum cholesterol was estimated within 72 hours of collection.

All the methods were checked for reliability, using varying concentrations of standards. Next, different concentrations of standard solutions were added to an aliquot of the sample to test the recovery of the standard added. Finally, different aliquots of the same sample were analysed separately and the agreement between the values obtained checked. In some cases where the samples had to be stored, aliquots of the sample were analysed immediately and after storage.

The chemicals used throughout the study were of research grade purity and obtained either from British Drug House Limited or Sarabhai Chemicals. All the solvents were distilled before use. The reagents used for various estimations are described in Table 7.

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TABLE 7 : Reagent and standards used for biochemical determinations.

Reagent /Standard	Mode of praparation
Ammonia solution	4 ml of 25% (GR) liquor ammonia solution was made upto 100 ml with distilled water.
Alkaline copper-tartrate	24g of anhydrous sodium carbonate and 12g of Rochelle salt were dissolved in 250 ml of water. To this 4g of copper sulphate dissolved in about 50 ml of water was added followed by 16g of sodium bicarbonate. The solution was stirred while preparing. 180g of anhydrous sodium sulphate was dissolved separately in 500 ml. of water, boiled to expel the air, cooled, mixed with the other solution and then was made upto one litre. The solution could be preserved at room temperature for 4 to 5 days and filtered if necessary before use.

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TABLE ____ (Contd.)

Reagent/Standard	Method of preparation
Arsenomolybdate reagent	25g of ammonium molybdate was dissolved in 450 ml of water and 21 ml of concentrated sulphuric acid slowly added with continuous stirring. To this 3g of disodium hydrogen arsenate dissolved in 25 ml of water was added. The reagent was mixed well and kept in an incubator at 37°C for 24-48 hours. The reagent was then stored in a glass stoppered bottle.
Alcoholic potassium hydroxide	2g of reagent grade potassium hydroxide was dissolved in 95% redistilled alcohol and diluted to 100 ml with alcohol. 10 ml of this stock was diluted to 50 ml with 95% alcohol on the day of use (0.4%).
Ammonium molybdate	5g of ammonium molybdate was dissolved in 10 N. sulphuric acid to a final volume of 100 ml.

TABLE (Contd.)

Reagent/Standard	Method of preparation
1-Amino-2-Naphthol 4-sulphonic acid (ANSA)	0.2g of aminonaphthol sulphonic acid, 2.4g sodium sulphite and 12.0g sodium metabisulphite were dissolved in glass distilled water and the volume made to 100 ml.
Acetic anhydride sulphuric acid	For every 20 ml of reagent grade, acetic anhydride, 1 ml of concentrated sulphuric acid was added with constant stirring. This reagent was made fresh every time just before use.
Barium hydroxide	Barium hydroxide was added to 500 ml of hot water and boiled for a few minutes (0.3N). It was allowed to cool, filtered, if necessary, stoppered and stored in a brown bottle.

Reagent/standard	Method of preparation
Calcium chloride	13.88 mg of calcium chloride ($CaCl_2$) was made upto 100 ml with water (0.05 mg/ml).
Chloroform-methanol mixture (2:1)	200 ml of chloroform and 100 ml of methanol were mixed and used.
Cholesterol standard	100 mg of cholesterol was dissolved in 100 ml of glacial acetic acid. 10 ml of this was then diluted to 100 ml with glacial acetic acid to give a concentration of 100 ug/ml.
Chromotropic acid	2.24g of the sodium salt of chromotropic acid was dissolved in 200 ml of glass distilled water. Separately 600 ml of concentrated sulphuric acid was added to 300 ml of glass distilled water cooled in ice. The cooled diluted acid was added to the cooled chromotropic acid solution. The solution was

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Reagent/standard	Method of preparation
	stored in brown bottles. The reagent was prepared fresh every 2 or 3 weeks.
Ferric Chloride 0.2% For Vitamin E	200 mg of Ferric Chloride in 100 ml of absolute alcohol and dissolved for estimation of Vitamin E
Glucose standard solution:	
(a) Stock	100 mg of pure glucose was dissolved in saturated benzoic acid solution and made upto 100 ml with benzoic acid solution.
(b) Intermediary standard	5 ml of the stock solution was diluted to 100 ml with benzoic acid solution. This contained 0.05 mg of glucose per ml.
(c) Working standard	1 ml of the intermediate stock glucose standard was made up to 4 ml with water. This contained

Reagent/standard	Method of preparation
	0.0125 mg/ml glucose.
Magnesium standard:	
(a) Stock	8.45 g of magnesium chloride was dissolved in distilled water and made up to one litre. This was used as stock magnesium standard.
(b) Working standard	1ml of the stock solution was diluted to 200 ml with water. This contains 5 ug/ml.
Phosphorus standard	87.87g of potassium dihydrogen orthophosphate (KH_2PO_4) was dissolved in 100 ml glass distilled water. 5 ml of this was taken and again made up to 100 ml with glass distilled water. 1 ml of this contained 10 mg phosphorus.

Reagent/standard	Method of preparation
Polyvinyl alcohol	0.2g of polyvinyl alcohol was weighed, transferred to a beaker and dissolved in about 5 ml of water with the aid of heat and stirring. The solution was made up to 100 ml with distilled water and a few drops of chloroform was added.
Sodium arsenite	6.5g of sodium arsenite was dissolved in 100 ml of glass distilled water (0.5M).
Sodium hydroxide	4g of sodium hydroxide pellets were dissolved in water and made up to 500 ml with water.
Sodium hydroxide (4N).	16g of reagent grade sodium hydroxide pellets were dissolved in distilled water and made up to 100 ml with distilled water.
Sodium metaperiodate	1.07g of sodium metaperiodate was dissolved in 100 ml of glass distilled water.

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Reagent/standard	Method of preparation
Sodium sulphite	2.52g of anhydrous sodium sulphite was taken in water and diluted to 100 ml with glass distilled water (0.2M).
10% Sodium tungstate	10g of sodium tungstate was dissolved in water and made up to 100 ml with distilled water.
2/3N Sulphuric acid	35g of concentrated sulphuric acid was added slowly with continuous stirring to a litre of distilled water. This was standardised against an alkali of known strength.
Sulphuric acid (10N)	69.5 ml of concentrated sulphuric acid was made up to 250 ml with glass distilled water.
Sulphuric acid (0.2N)	3 ml of concentrated H_2SO_4 was diluted to 500 ml with glass distilled water.

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Reagent/standard	Method of preparation
Sulphate-sulphite reagent	20.8g of anhydrous sodium sulphite, 7.9g of sodium sulphate, 90 ml of acidified water containing 0.2 ml of concentrated sulphuric acid were mixed and made up to 100 ml with water. The pH was adjusted to above 7.0 and stored at room temperature.
Titan yellow	0.1g of the powdered titan yellow was added to 200 ml of distilled water (0.05M).
Tripalmitin standard	120 mg of tripalmitin was dissolved in 100 ml of chloroform.1 ml of this solution was again dissolved in 10 ml of chloroform to give a standard solution of 12 µg in 0.1 ml.
Vitamin E standard	
(a) Stock	1g of vitamin E acetate was taken in a flask to which a reflexer was fixed. 0.25g of pyrogallol, 1 ml of potassium hydroxide

Reagent/standard	Method of preparation
	(60g KOH pellets dissolved in 40 ml distilled water) and 5 ml of 96% alcohol were added to the flask. The sample was saponified by reflexing over a water bath for 10 minutes using reflex condenser, cooled and transferred to a separating funnel. The flask was rinsed with approximately 100-150 ml petroleum ether and the washings added to the funnel. The residue was washed with 500 ml distilled water in order to remove the alcohol and potassium hydroxide. The water layer (bottom layer) was discarded. The sample (top layer) was next passed through a 2-3 cm (1.0 cm diameter) long column of anhydrous sodium sulphate and the elute evaporated to a known volume (approx. 25 ml) in a rotary evaporator at 60°C. The final volume was made to 100 ml with petroleum ether (40-60°C). This formed the stock containing 0.9111g of Vitamin E/dl.

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Reagent/Standard	Method of preparation
(b) Working vitamin E standard	0.1 ml of stock solution was diluted to 10 ml with petroleum ether (40-60°C), 2 ml of this were diluted to 10 ml and 1, 2, 3 and 4 ml aliquots from this were made up to 4 ml each to get working standard solutions (containing 0.2-1 mcg of vitamin E).
Zinc sulphate	5g of zinc sulphate was dissolved in 100 ml of distilled water.
α'-α-dipyridyl	0.2g of α', α'-dipyridyl was dissolved in 100 ml of 3% glacial acetic acid (v/v).

ANALYTICAL PROCEDURES

Methods used for biochemical analysis are summarized in Table 8 .

(A) Blood Hemoglobin : Blood hemoglobin was determined according to the oxyhemoglobin method of Evelyn and Malloy (1938). Twenty microlitres (20 ul) of blood were collected in a hemoglobin pipette and transferred to a test tube containing 8 ml of 1% ammonia solution. The colour developed was read immediately in a Klett-Summerson colorimeter using green filter (540 nm). The colour was read against a blank containing 1% ammonia solution. The results were expressed as grams per decilitre of blood.

(B) Blood Glucose : Blood sugar was estimated according to Nelson-Somogyi method (1952). Twenty microliters of blood were taken (20 ul) and mixed in 0.7 ml of distilled water (in duplicate). To this, 0.04 ml of barium hydroxide and 0.4 ml of zinc sulphate were added, shaken well and kept for a minute after which the contents were centrifuged and 0.5 ml of the filtrate was taken in Folin Wu tube and made up to 1 ml with distilled water. The standard glucose solution (0.5 ml) was treated similarly. The tube containing distilled water

instead of the sample or standard served as a blank. One ml of alkaline copper tartrate was added to all the three tubes which were then kept in a boiling water bath for 10 minutes. The tubes were quickly cooled in iced water for a minute and 0.5 ml of arsenomolybdate reagent was added. The tubes were shaken well and the volume made up to 5 ml by adding 2.5 ml of distilled water. The tubes were again shaken well, the contents taken in spectrophotometric cuvettes and read against the balnk at 680 nm in a spectrophotometer.

Table 8: Biochemical parameters used in the investigations.

Parameters	Method used
A) Blood:	
1. Blood hemoglobin	Oxyhemoglobin (Evelyn and Malloy, 1938).
2. Blood glucose	Nelson-Somogyi (Somogyi, 1952)
B) Serum:	
1. Cholesterol	Acetic Anhydride method (Sperry and Webb, 1950)
2. Phospholipid	Bartlett (1956)
3. Triglyceride	Van Handel and Zilversmith (1957)
4. Vitamin E	α — α-dipyridyl method (Qwaife et al., 1949)
5. Magnesium	Neil and Neely (1956)

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Serum lipids :

Extraction of lipids from serum - The lipids in the serum were extracted according to the method of FOLCH et al (1957)

0.5 ml of the serum was taken into a 2.5 ml capacity glass stoppered test tube. To 10 ml of 2:1 v/v chloroform-methanol mixture were added to the serum with constant and vigorous swirling during addition. The tube was stoppered and sealed with a drop of water and shaken gently with a swirling motion for one minute. The sample was allowed to stand at room temperature with occasional gentle swirling for one hour after which an equal volume of distilled water was added gently down the side of the tube. The tube was then stoppered without further shaking and allowed to stand in the refrigerator over night or longer (1-4 days) till the upper aqueous phase containing the impurities in the original mixture separated out. The lower chloroform phase was removed through a long cannula as completely as possible by passing the cannula through the aqueous phase and the protein precipitated down the wall to the bottom of the tube. The amount of chloroform extract thus obtained was measured. The extract was taken into a separate stoppered test tube and used for the determination of serum lipids.

Phospholipid - Phospholipid was estimated according to the method of Bartlett (1956). An aliquot of 0.5 ml of the lipid extract prepared as above was evaporated at 40-50°C. To this was added 1 ml of perchloric acid (60%) and the sample digested on a sand bath for 1 hour at 230°C. After digestion, the volume was again made up to 1 ml with perchloric acid. This was followed by the successive additions of 8.1 ml of water, 0.5 ml of 5% ammonium molybdate solution and 0.4 ml of aminonapthol sulphonic acid (ANSA). The contents were mixed thoroughly on a vortex mixer and the tubes kept in a boiling water bath for 10 minutes, cooled to room temperature and the colour read at 820 nm against a blank. Standard containing different amounts of phosphorus (from 1 to 12 ug) were treated in the same way. Total phospholipid was calculated by multiplying lipid phosphorus by 25.

Total cholesterol - Estimation of total cholesterol was carried out according to the method of Sperry and Webb (1950). Aliquots of the lipid extract (0.5 ml) were taken in duplicate and evaporated to dryness at 40 to 50°C. Two ml of glacial acetic acid and 2 ml of acetic anhydride-sulfuric acid reagent were added and the tubes shaken thoroughly. After keeping in the dark for 20 minutes the colour developed was read in a spectrophotometer at 650 nm

against acetic acid blank. Different aliquots of (0.5, 1.0, 1.5, 2.0, 2.5 and 3 ml) standard cholesterol (100-300 µg) were taken and treated in the same way as the sample. Essentially the same procedure was followed for the estimation of milk cholesterol.

Triglyceride - Triglyceride was estimated according to the method of Van Handel and Zilversmith (1957). Aliquots of 0.2 ml of lipid extract were taken in duplicate and evaporated to dryness at 40-50°C. To one of the tubes 0.5 ml of distilled alcohol was added and to the second 0.5 ml of alcoholic KOH was added. The tubes were then kept at 60-70°C in a water bath for 15 min after which 0.5 ml of 0.2N H_2SO_4 was added. The tubes were kept in a boiling water bath for 15 minutes or till the tubes were free from alcohol. They were then taken out, 0.1 ml of sodium metaperiodate solution added and they were allowed to stand for 10 minutes. Now 0.1 ml of sodium arsenite solution and 5 ml of chromotropic acid were then added in succession. The contents were mixed well and again kept in a boiling water bath for 30 minutes covering the tubes with glass marbles. The tubes were then cooled to room temperature and the colour developed read at 570 nm against blank containing alcohol instead of sample. Tripalmitin was used as standard and different

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aliquots of the same were treated in the same way as the sample. The concentration of standard ranged between 12 to 72 µg.

Magnesium - Serum magnesium was estimated according to the method of Neil and Neely (1956). Aliquots of 0.2 ml of serum were treated with 1 ml of distilled water followed by 0.4 ml of 10% sodium tungstate and 0.4 ml of 2/3N sulfuric acid. The precipitated protein was then centrifuged and 1 ml of the supernatant was transferred to another test tube. To this were added 0.2 ml of distilled water, 0.2 ml of polyvinyl alcohol and 0.2 ml of titan yellow and the contents were mixed. Finally, 0.4 ml of 4N sodium hydroxide was added and the colour developed was read at 520 nm in a spectrophotometer. At the same time put up 0.2 ml of calcium chloride and 0.8 ml of water and 0.2 ml of calcium chloride, standard and water (to make up to 1 ml) for use as blank and standard respectively completing these in the same way as the test. Read standard and unknown against the blank using a green filter or with the instrument set at 520 nm.

The variation in magnesium concentration in standards was achieved using 0.2 to 1.0 ml aliquots of working standard solution containing 5 ug/ml of magnesium and making them to a volume of 1 ml.

Vitamin E:

This was estimated by the method of Quaife, Scrimshaw and Lowry (1949). To 0.6 ml of serum were added 0.6 ml of absolute alcohol and 3.0 ml of petroleum ether (40 - 60°c). The contents were mixed thoroughly and centrifuged. 2 ml of supernatant were taken in another tube and vacuum dried at 60°c. To the residue, 2.5 ml of absolute alcohol, 0.3 ml of 0.2 per cent α-α dipyridyl and 0.3 ml of 0.2 per cent ferric chloride solution were added and the colour developed read after 90 seconds at 520 nm in a spectrophotometer against a blank containing 2.5 ml alcohol, 0.3 ml of α-α -dipyridyl and 0.3 ml ferric chloride.