CHAPTER III

METHODS AND MATERIALS

A formative research on the prevalence of NAFLD in type 2 diabetics was conducted. Propagation of lifestyle modification through nutrition counselling was the subsequent phase of the research. A thorough investigation of quality of life was carried out on the NAFLD subjects. A detailed estimation of the knowledge attitude and practices was carried out on the NAFLD patients' pre and post lifestyle modification counselling. A plant with anti-dyslipidemic and hypoglycemic potential is tinospora cordifolia. With no clinical studies after showing commendable impact on the glycemic and lipemic profile in animal models, the last phase of the study comprised of supplementing tinospora cordifolia pure stem extract to patients with diabetic dyslipidemia. With the above mentioned background, the research was carried out in the following sequential manner:

PHASE I

- **Phase I (A):** Prevalence of NAFLD in association with cardio-metabolic risk factors among type 2 diabetes patients.
- **Phase I (B):** Quality of life of type 2 diabetes patients with NAFLD.

PHASE II

- **Phase II (A):** Development of lifestyle modification therapy module for the management of type 2 diabetes and NAFLD.
- **Phase II (B):** Impact of nutrition counselling on the knowledge attitude and practices of type 2 diabetes patients with NAFLD.
- **Phase II (C):** Impact of lifestyle modification therapy in the management of NAFLD among type 2 diabetes patients.

PHASE III

- **Phase III (A):** Qualitative phytochemical analysis of tinospora cordifolia stem
- **Phase III (B):** Impact of tinospora cordifolia pure stem extract supplementation in the management of diabetic dyslipidemia

PHASE I (A): PREVALENCE OF NON-ALCOHOLIC FATTY LIVER DISEASE (NAFLD) IN ASSOCIATION WITH CARDIO-METABOLIC RISK FACTORS IN PATIENTS WITH TYPE 2 DIABETES MELLITUS

A formative research in a clinical set up was conducted to map the prevalence of NAFLD in type 2 diabetes patients (fig 3.1). The study was approved by the Institutional Medical Ethics Committee (IECHR/2012/17) of the Maharaja Sayajirao University of Baroda.

The specific objectives of this phase were:

- To map the prevalence of NAFLD in type 2 diabetic patients
- To study the association of NAFLD with metabolic syndrome
- To assess the cardio-metabolic profile of NAFLD patients with type 2 diabetes
- To determine relationship between diet and NAFLD
- To assess the link between physical activity status and NAFLD
- To identify predictor variables of NAFLD in type 2 diabetes
- To assess the possibility of fibrosis amongst NAFLD patients.

Sample Size

An estimate of ~70-75% has been made about the occurrence of some form of NAFLD in type 2 diabetics (Medina et al., 2004) with 50-75% demonstrating fatty infiltration upon ultrasound (Loguercio et al., 2001; Teli et al., 1995).

The formula for calculating the sample size was (Gorstein et al., 2007)

$$N = \frac{(Z\ alpha)^2\ (p*q)}{d2}$$

wherein,

Z alpha = The value of z from the probability. If the values are normally distributed, then 95% of the values will fall within 2 standard errors of the mean. The value of z corresponding to this is 1.96.

p = Prevalence of the health condition under consideration (NAFLD). The value of p was taken as 62.5% (an average of 50-75% was obtained because about 50-75% of the type 2 diabetics demonstrate presence of NAFLD upon ultrasound).

q = 100 – p

d = Precision of the estimate. The relative precision is taken as the proportion of p and the maximum permissible limit is 20% of p. Thus, 20% of 62.5% is 12.5, which was taken as d.

Substituting the values in the formula,

$$N = \frac{(1.96)^2 [62.5 (100 - 62.5)]}{(12.5)^2}$$

$$N = \frac{3.84 * (62.5 * 37.5)}{156.25}$$

Therefore, N = 57.6.

However, 105 type 2 diabetics aged between 30-75 years were voluntarily enrolled from a diabetic clinic from January, 2013 to May, 2013 for participation in the study based on the below mentioned selection criteria.

Selection Criteria

A strict criterion for inclusion was complete abstinence from alcohol so as to characterize fatty liver of non-alcoholic nature. Being a resident of Baroda city, willingness to participate were the other inclusion criteria. Patients with known liver disease (viral, autoimmune and genetic), consuming hepatotoxic drugs (gluco-corticoids, synthetic oestrogens, high dose oestrogen, tamoxifen, calcium channel blockers and methotrexate), those with recent gastrointestinal surgery (jejunoilial bypass or extensive small bowel resection), type 1 diabetes and gestational diabetes were excluded from the study.

Methodology

After the detailed explanation of the study protocol to the subjects, the 105 type 2 diabetes patients consented to be a part of the study (appendix 1). Data on medical history, family history of NCDs, drug profile, supplements usage and addiction patterns was obtained with the help of a semi-structured pre-tested questionnaire

(appendix 2). Anthropometric measurements like weight, height, waist circumference and hip circumference were taken and subsequently waist hip ratio, waist stature ratio and abdominal volume index were calculated for assessment of the nutritional status. Blood pressure of the subjects was also estimated with the help of a mercury sphygmomanometer. Details regarding the diet were obtained through the 24 hour dietary recall method. Physical activity status was assessed with the help of the international physical activity questionnaire, short form. Ten ml of blood after 12 hours of overnight fasting was drawn by a trained lab technician from Thyrocare for liver function test, hepatitis B surface antigen, hepatitis C antibody, kidney function test, complete blood count, iron profile, lipid profile, thyroid profile, vitamin D, glycated hemoglobin and hs-CRP. Two of the study subjects were diagnosed with hepatitis B surface antigen and another one with hepatitis C antibody and were excluded from the study but were asked to consult a hepatologist at the earliest. Seven other subjects withdrew from the study post biochemical analysis for reasons such as; unavailability of time for visiting the radiologist (n=1), perception of futility of ultrasound (n=1) and fear of getting diagnosed with NAFLD (n=5). The abdominal ultrasound was performed by an experienced radiologist who was blinded to the medical background and history of the subjects. The maximum period of difference between conduction of blood test and that of ultrasound was less than a week. A total of 95 type 2 diabetes patients underwent abdominal ultrasound.

Data was generated on the following parameters:

1. **Self reported data:** Family history of NCDs, medical history, drug profile, supplement usage, addiction patterns and physical activity.
2. **Anthropometric data:** Weight, height, waist circumference, hip circumference, waist hip ratio, waist stature ratio and abdominal volume index
3. **Bio-physical data:** Blood pressure
4. **Dietary data:** 24 hour dietary recall
5. **Bio-chemical data:** Hepatitis B surface antigen, hepatitis C anti-body, complete blood count (total lymphocyte count, neutrophils, lymphocyte, monocytes, eosinophils, basophils, total RBC, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, RDW-CV, platelet count, platelet distribution

width, mean platelet volume), iron profile (iron, total iron binding capacity, transferrin saturation, ferritin), kidney function test (blood urea nitrogen, creatinine, uric acid, calcium), Lipid profile (total cholesterol, HDL-C, LDL-C, triglycerides, VLDL-C), hs-CRP, liver function test (alkaline phosphatase, bilirubin direct, bilirubin total, bilirubin indirect, gamma glutamyl transferase, aspartame aminotransferase, alanine aminotransferase, total protein, albumin), thyroid function (tri-iodothyronine, thyroxine, thyroid stimulating hormone), vitamin D (25 OH Vitamin D) and glycosylated hemoglobin.

6. **Imaging data:** Abdominal ultrasound (liver scan).

PHASE I (B): QUALITY OF LIFE OF TYPE 2 DIABETES PATIENTS WITH NON-ALCOHOLIC FATTY LIVER DISEASE

The study was conducted to assess the quality of life of type 2 diabetes patients with confirmed NAFLD from the gender and grades of hepatic steatosis perspective.

The criterion for inclusion in the research was confirmed diagnosis of NAFLD amongst type 2 diabetics who had participated in the previous phase of the study. Thus, a total of 74 type 2 diabetes patients aged between 30-75 years were enrolled from the first phase of the research.

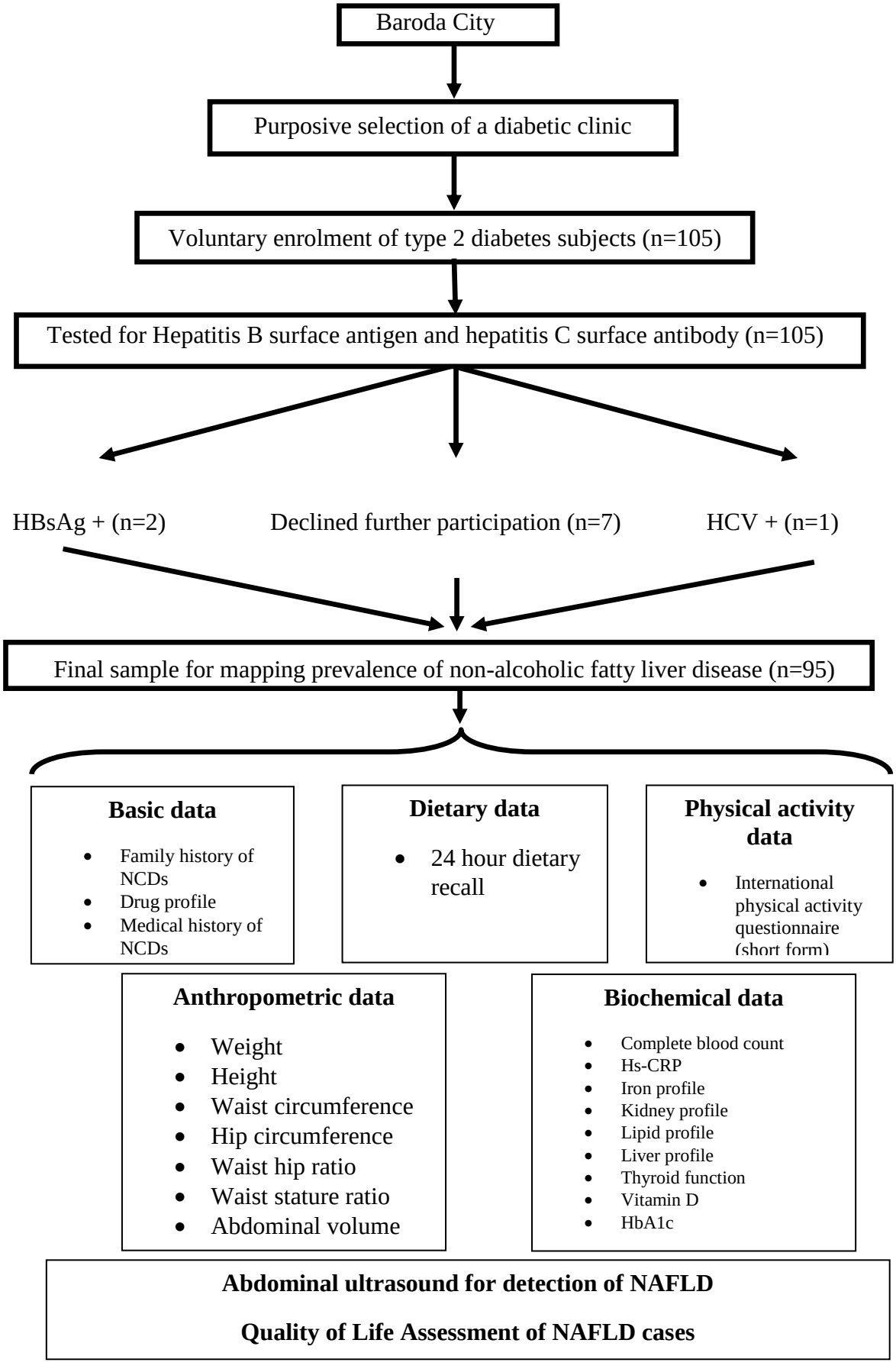
Instrument for Assessment

The Quality of Life Instrument for Indian Diabetes Patients (QOLID) was used to assess the quality of life. QOLID**,** is a tool especially developed for the Indian diabetes patients, which comprises of a set of 34 items (questions) representing eight different domains of quality of life. It is a reliable and valid tool for assessment of quality of life of Indian patients with diabetes. It has an Overall Cronbach's Alpha value of 0.894 (subscale- 0.55 to 0.85) showing high internal consistency and good responsiveness to metabolic control and co-morbidities establishing discriminant validity. The psychometric strength of the questionnaire is further enhanced by the use of a standard Likert scale across all questions with ratings from one to five (Nagpal et al., 2010). The detailed questionnaire is given in appendix 3.

Methodology

Of the 74 type 2 diabetes patients who were diagnosed with NAFLD, all gave their written and informed consent for participation in assessment of quality of life of type. A time of convenience was obtained from the patients to obtain information about the said profile on the following eight domains; role limitations due to physical health, physical endurance, general health, treatment satisfaction symptom frequency, financial worries, mental health and diet advice satisfaction.

FIG 3.1 EXPERIMENTAL DESIGN PHASE I



PHASE II (A) DEVELOPMENT OF LIFESTYLE MODIFICATION THERAPY MODULE FOR THE MANAGEMENT OF NAFLD IN TYPE 2 DIABETES

A booklet on the management of type 2 diabetes mellitus and NAFLD was developed in English and Gujarati for the management of NAFLD in type 2 diabetes based on the principles of nutritional management of NAFLD and physical activity recommendations (discussed in the chapter of review of literature). The booklet was provided to all the NAFLD patients in the experimental arm as a ready reckoner. Taking the medical ethos into consideration, the controls who were the recipients of standard care alone (discussed later), were also provided with the booklet at the termination of the study. Following aspects were covered in the booklet:

- Type 2 diabetes mellitus
    - Risk factors
    - Symptoms
    - Diagnosis
    - Important factors for diabetes management
    - Possible complications arising out of diabetes
- Non-alcoholic fatty liver disease
    - Risk factors
    - Predisposition to NAFLD due to diabetes
    - Indicators of fat deposition in liver and diagnosis
    - Why treatment of NAFLD is necessary
    - Treatment modalities of NAFLD
    - Guidelines for dietary management of NAFLD and diabetes
    - Importance of physical activity in treatment of NAFLD

The booklet as slides (English version) is projected in appendix 4.

PHASE II (B AND C): IMPACT OF NUTRITION COUNSELLING ON KNOWLEDGE ATTITUDE AND PRACTICES OF TYPE 2 DIABETES PATIENTS WITH NAFLD AND IMPACT OF LIFESTYLE MODIFICATION THERAPY IN THE MANAGEMENT OF NON ALCOHOLIC FATTY LIVER DISEASE AMONG TYPE 2 DIABETES PATIENTS

It was hypothesized that nutrition counselling propagating lifestyle modification will bring about favourable changes in the metabolic profile of type 2 diabetics with NAFLD and also aid in improving the KAP. This phase of the study was carried out to achieve the below mentioned objectives:

- To counsel the NAFLD patients to adopt lifestyle modification therapy.
- To measure the impact of counseling propagating lifestyle modification in type 2 diabetic NAFLD subjects in terms of:
    - Anthropometry
    - Dietary profile
    - Physical activity status
    - Lipemic status
    - Glycemic status
    - Liver enzymes status
    - Inflammation status
    - Metabolic syndrome status
    - Liver Status

The study was approved (IECHR/2013/6) by the Institutional Medical Ethics Committee of the Department of Foods and Nutrition, The Faculty of Family and Community Sciences, The Maharaja Sayajirao University of Baroda. Informed, written and understood consent was obtained from the study subjects (appendix 5).

Selection Criteria

The confirmed cases of NAFLD were voluntarily enrolled from phase one of the study. Based on their willingness for participation for the subsequent four months and their availability in Baroda, they were enrolled for the study (n=60) on the basis of informed, understood and written consent to take part in this research.

Phase II (B) Methodology

A thorough assessment about the knowledge attitude and practices (KAP) about type 2 diabetes and NAFLD was carried out on confirmed cases of NAFLD who were enrolled from the first phase of the study (n=60) with the help of a pre-tested questionnaire (appendix 6). The subjects were randomly allocated into two arms; experimental group (n=30) and control group (n=30). The questionnaire consisted of three major sections; knowledge about type 2 diabetes, knowledge about NAFLD and attitudes and practices about type 2 diabetes and NAFLD. It consisted of close ended multiple choice responses or simple yes or no responses. For each correct response a score of one was assigned. Following were the components that were assessed under the KAP survey:

a) Knowledge pertaining to diabetes: Definition, risk factors, symptoms, diagnosis, impact of diabetes on other organs and effective management (table 3.1)

b) Knowledge pertaining to NAFLD: Awareness, definition, risk factors, pre-disposition to NAFLD owing to diabetes, pathophysiology and progression of NAFLD, symptomatology, diagnosis, need for addressing NAFLD, treatment modalities (table 3.2)

c) Attitudes and practices: Satisfaction with current exercise regime, dietary restrictions while eating, regularity in medicine consumption, visits to the doctor for health check up, monitoring of fasting blood glucose, monitoring of glycosylated hemoglobin, monitoring of blood pressure, monitoring of lipid profile and kidney profile, visit to the ophthalmologist and abdominal ultrasound for liver scan (table 3.3).

Knowledge attitude and practice index: For all the correct answers, a score of one was assigned and a wrong answer was scored as zero. Similarly, questions having a multi-choice nature where multiple suggested options were correct were each assigned a score of one. For the above mentioned parameters of assessment of KAP, the scores enlisted in the tables were assigned and a total KAP score was further categorised as low, average or high (table 3.4).

TABLE 3.1: KNOWLEDGE INDEX OF TYPE 2 DIABETES MELLITUS

Thrust areas	Minimum score	Maximum score
Definition of diabetes	0	3
Risk factors for diabetes	0	10
Symptoms of diabetes	0	8
Diagnosis of diabetes	0	4
Impact of diabetes on other organs	0	6
Effective management of diabetes	0	4
Total Score	**0**	**35**

TABLE 3.2: KNOWLEDGE INDEX OF NAFLD

Thrust areas	Minimum score	Maximum score
Awareness about NAFLD	0	1
Definition of NAFLD	0	1
Risk factors for NAFLD	0	6
Predisposition to NAFLD due to diabetes	0	2
Occurrence and progression of NAFLD	0	3
Symptoms of NAFLD	0	3
Diagnosis of NAFLD	0	2
Relevance of treatment of NAFLD	0	3
Treatment modalities of NAFLD	0	5
Total score	**0**	**26**

TABLE 3.3: ATTITUDE AND PRACTICE INDEX ON TYPE 2 DIABETES AND NAFLD

Thrust areas	Minimum score	Maximum score
Satisfaction with current exercise regime	0	1
Dietary restrictions while eating	0	3
Regularity of medication	0	1
Regularity of health check up	0	1
FBS monitoring	0	1
HbA1c monitoring	0	1
BP monitoring	0	1
Lipid profile monitoring	0	1
Kidney profile monitoring	0	1
Eye monitoring	0	1
Abdominal ultrasound	0	1
Total score	**0**	**13**

TABLE 3.4: CLASSIFICATION AND CATEGORY OF KAP SCORES

CATEGORY	PERCENT SCORE
Poor	<25
Average	25-50
Good	50-74

Phase II (C) Methodology

After assessment of the knowledge attitude and practices (KAP) of the type 2 diabetic subjects with NAFLD from the experimental group and control group, information regarding medical history, family history of NCDs, drug profile was obtained from the subjects with the help of a semi-structured questionnaire (appendix 7). The anthropometric data comprising of weight, height, waist circumference, hip circumference was also taken to assess the nutritional status. Details of the diet were elicited through the 24 hour dietary recall method. Physical activity patterns were assessed with the help of the international physical activity questionnaire, short form. Bio-chemical assessment after 12 hours overnight fast was done to estimate; liver function test, lipid profile, kidney function test, hs-CRP, HbA1c and thyroid profile. An abdominal ultrasound was performed by an experienced radiologist who was blinded to the medical profile of the subjects and liver span was also assessed (fig 3.2).

The experimental arm received nutrition counselling propagating lifestyle modification in addition to standard care whereas; the control arm only received standard care. Nutrition counselling to adopt lifestyle modification therapy was carried out for a period of four months with a personal monthly visit to each of the participant. The dietary changes to be incorporated and physical activity recommendations were individualised based on the nutritional status and the co-morbid conditions. Standard care had to be tailor made for each of the subjects.

At the end of each monthly follow up data on anthropometry, 24 hour dietary recall, blood pressure and physical activity was obtained for the experimental arm as well as the control arm (table 3.5). At the end of four months, biochemical profile assessment followed by an abdominal ultrasound was performed to assess the impact of lifestyle modification therapy on NAFLD patients. For weight loss through lifestyle modification, techniques such as goal setting, self monitoring, stimulus control, alternative behaviours, problem solving and cognitive re-structuring (Bellentani et al., 2008) were used to achieve the desired impact.

Data Monitoring, Management and Analysis

The data was collected by the researcher herself using a pre tested questionnaire and the blood samples were collected by a trained technician from an accredited lab 'Thyrocare'. The biomedical waste generated was discarded appropriately as per the standard norms. Follow up with the subjects of the intervention arm was maintained telephonically on a weekly basis and along with personal visits on a monthly basis for a period of four months. The data was analysed with the help of SPSS. All the data both in hard copies (questionnaires and consent forms) and soft copy was kept at a central and safe place and was accessible at all times.

FIG 3.2 EXPERIMENTAL DESIGN PHASE II (B,C)

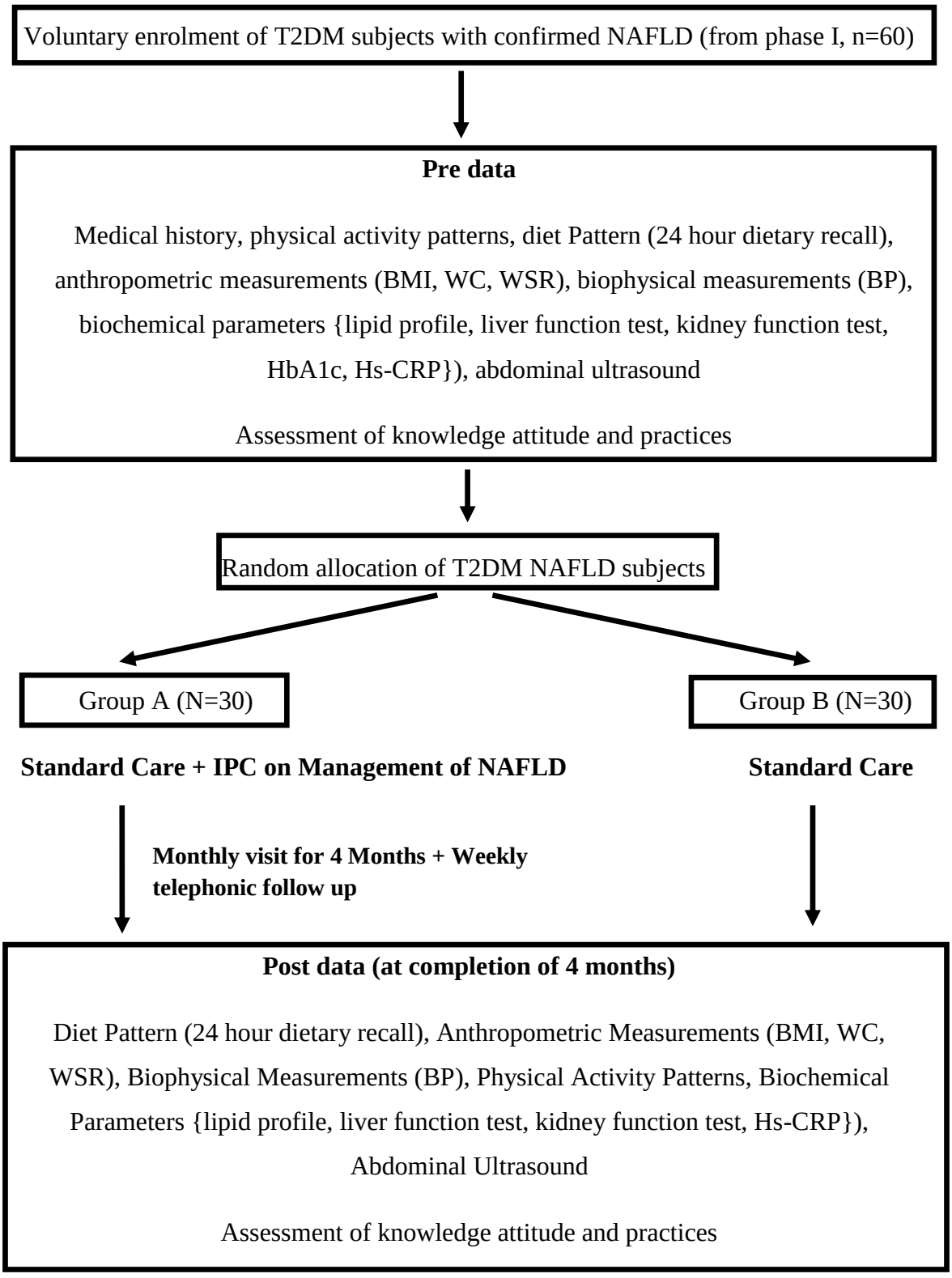


TABLE 3.5: DATA OBTAINED IN PHASE II

TYPE OF DATA	PRE DATA	POST DATA			
		1st month	2nd month	3rd month	4th month
General information	√	-	-	-	-
Medical history	√	-	-	-	-
Anthropometric data	√	√	√	√	√
Blood pressure	√	√	√	√	√
24 hour dietary recall	√	√	√	√	√
Biochemical profile	√	-	-	-	√
Physical activity	√	√	√	√	√
Abdominal ultrasound	√	-	-	-	√
Knowledge attitude and practices	√	-	-	-	√

PHASE III (A): PHYTOCHEMICAL ANALYSIS OF TINOSPORA CORDIFOLIA

The objective of this phase was to determine the qualitative phytochemical profile of tinospora cordifolia stem (Guduchi capsule, Himalaya, Herbal Health Care product).

The marketed Guduchi drug that contains mature stem of tinospora cordifolia was procured from the medical representative of Himalaya. It was analysed for the presence of the following compounds: tannins, alkaloids, saponins, flavonoid, steroids, terpenoids and cardiac glycosides. The sample was sent for analysis to the Food Testing Laboratory, Indian Institute of Crop Processing Technology, Ministry of Food Processing Industries, Govt. of India, Thanjavur.

Analysis of Samples

The given sample was extracted with oil and the methylated extract was analyzed through GC-MS for the identification of different compounds.

GC Programme

Column: Elite-5MS (5% Diphenyl / 95% Dimethyl poly siloxane), 30 x 0.25mm x 0.25microm df
Equipment: GC Clarus 500 Perkin Elmer
Carrier gas: 1ml per min, Split: 10:1
Detector: Mass detector Turbo mass gold-Perkin Elmer
Software: Turbomass 5.2
Sample injected: 2 microlitre

Oven temperature Programme

110° C -2 min hold
Up to 200° C at the rate of 10 ° C/min-No hold
Up to 280 ° C at the rate of 5° C / min-9 min hold
Injector temperature 250° C
Total GC running time 36 min

MS Programme

Library used NIST Version-Year 2005

Inlet line temperature 200° C

Source temperature 200 ° C

Electron energy: 70 eV

Mass scan (m/z): 45-450

Solvent Delay: 0-2 min

Total MS running time: 36 min

PHASE III (B): IMPACT OF TINOSPORA CORDIFOLIA SUPPLEMENTATION IN THE MANAGEMENT OF DIABETIC DYSLIPIDEMIA

Tinospora cordifolia has shown potential in the management of dyslipidemia and dysglycemia in animal models. However, there are no clinical studies to corroborate the same. For the present study it was hypothesized that tinospora cordifolia pure stem extract may have anti-dyslipidemic, hypoglycemic and hepato-protective properties. The objective of the study was to assess the impact of tinospora cordifolia stem pure extract supplementation on anthropometric, glycemic, lipemic and metabolic syndrome profile of subjects with diabetic dyslipidemia.

Supplementation Dosage

A dosage of 500 mg per day is considered to be safe for consumption (Upadhyay et al., 2010). 250 miligrams of mature stem extact of tinospora cordifolia was encapsulated in gelatin coated capsules. Very less information on toxicity is available (Gupta et al., 2003) and neither negative herb-drug interaction has been reported. From the nutrient drug interaction perspective, it has a potential basis for clinically desirable drug interactions (Lather et al., 2011) because of which it is considered desirable as a supportive drug for glycemic control, avert complications and alleviate side effects of synthetic drugs (Sharma et al., 2015).

The study was approved by the Institutional Medical Ethics Committee of the Department of Foods and Nutrition, Faculty of Family and Community Sciences, The Maharaja Sayajirao University of Baroda (IECHR/2013/5).

Selection Criteria

A must criterion was the presence of diabetic dyslipidemia diagnosed on the basis of recent secondary data and subjects who were on OHA and recently begun on statin or were to be prescribed low dose statin. Care was taken to include only subjects of Baroda city and those who had no apparent complications. Further, those willing to consume two Guduchi capsules (Himalaya Herbal Health Care product, 1 capsule=250mg tinospora cordifolia) twice everyday pre meal (for experimental group for a period of 60 days) were enrolled. It would have been highly unethical to

bar a dyslipidemic patient of known treatments available to manage and control dyslipidemia because of which subjects to be on statin therapy were included in both the arms.

Methodology

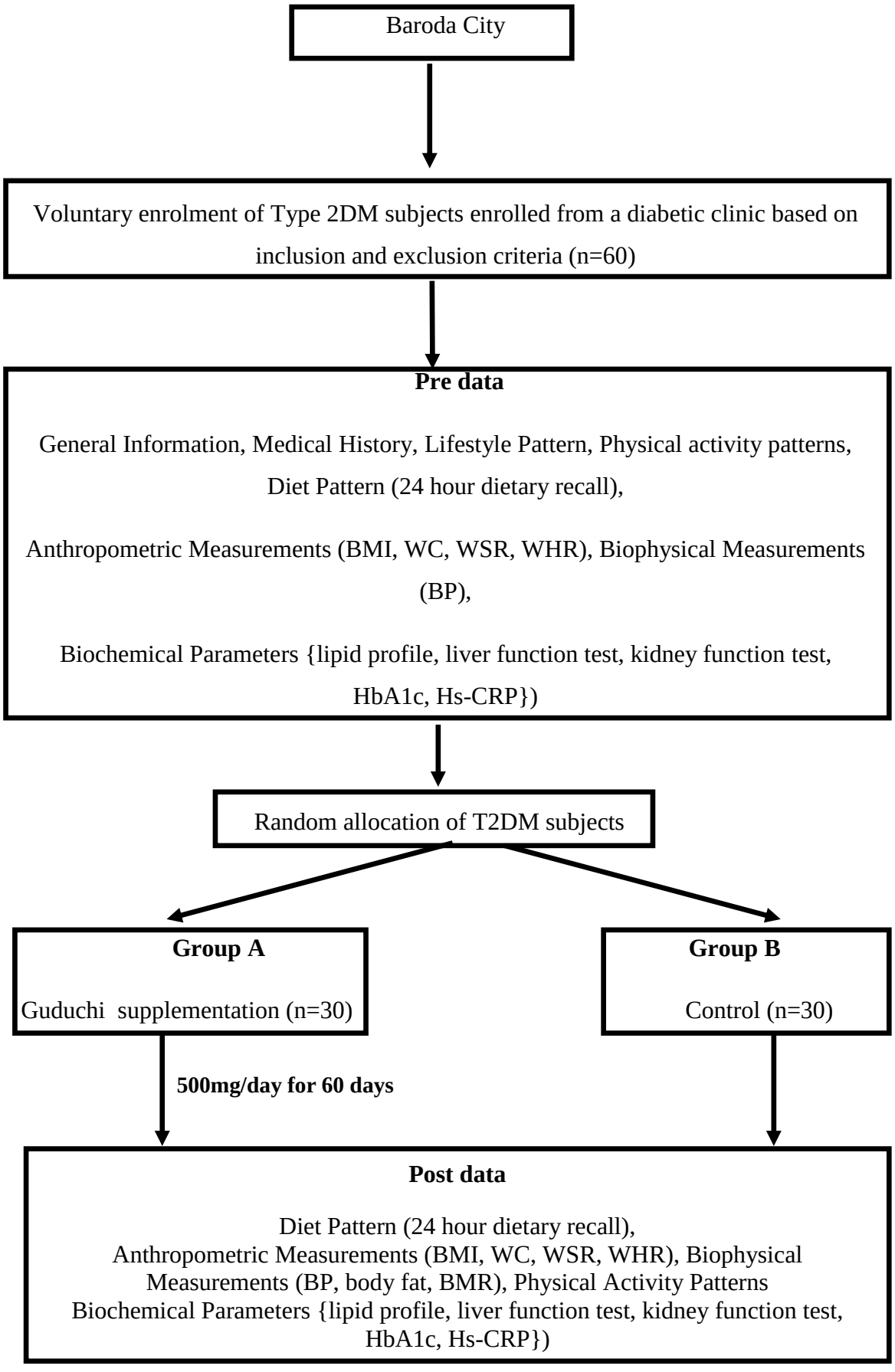
Written, informed and understood consent was obtained from the subjects (appendix 8) (n=60, expecting a 15% reduction in the mean triglyceride values after supplementation, the sample size is adequate). On enrolment, baseline data on medical history, family history of lifestyle diseases, and duration of diabetes, drug profile, anthropometric data, dietary data and physical activity data was obtained with the help of a semi-structured questionnaire (appendix 9). Ten ml of 12 hours overnight fasting blood was drawn to estimate liver function test, kidney function test, haemoglobin, thyroid, lipid profile and hs-CRP. Parallely, they were assigned into either of the two groups; wherein, Group A (Guduchi capsule, Himalaya, Herbal Health Care product supplementation, n=30) that was asked to consume two Guduchi capsules (One capsule= 250mg tinospora cordifolia), pre meal for a period of 60 days, along with the prescribed OHA and statin (fig 3.3). If the subjects reported any allergic reactions or any unpleasant experience after consuming the tablets, they were asked to discontinue the consumption (n=1, reported lower GIT disturbances and hence was excluded from the study). Group B (n=30) served as the control group and they were asked to continue with their OHA and statin. After two months of intervention, the anthropometric profile, diet patterns, physical activity profile, biochemical profile were re-assessed to analyse the impact of the intervention.

Parameters on Which Data Was Gathered

1. **Self reported data:** Family history of NCDs, medical history, drug profile, supplement usage, addiction patterns and physical activity.
2. **Anthropometric data:** Weight, height, waist circumference, hip circumference, waist hip ratio, waist stature ratio and abdominal volume index
3. **Bio-physical data:** Blood pressure
4. **Dietary data:** 24 hour dietary recall
5. **Bio-chemical data:** Hepatitis B surface antigen, hepatitis C anti-body, complete blood count (total lymphocyte count, neutrophils, lymphocyte,

monocytes, eosinophils, basophils, total RBC, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, RDW-CV, platelet count, platelet distribution width, mean platelet volume), iron profile (iron, total iron binding capacity, transferrin saturation, ferritin), kidney function test (blood urea nitrogen, creatinine, uric acid, calcium), Lipid profile (total cholesterol, HDL-C, LDL-C, triglycerides, VLDL-C), hs-CRP, liver function test (alkaline phosphatase, bilirubin direct, bilirubin total, bilirubin indirect, gamma glutamyl transferase, aspartame aminotransferase, alanine aminotransferase, total protein, albumin), thyroid function (tri-iodothyronine, thyroxine, thyroid stimulating hormone), vitamin D (25 OH Vitamin D) and glycosylated hemoglobin.

FIG 3.3 EXPERIMENTAL DESIGN PHASE III (B)



Data Monitoring, Management and Analysis

The data was collected by the researcher herself using a pre tested questionnaire and the blood samples were collected by a trained technician from an accredited lab 'Thyrocare'. The biomedical waste generated was discarded appropriately as per the standard norms. The data was entered into Microsoft excel spread sheets and then subjected to appropriate statistical analysis using Microsoft Excel data analysis package for calculating mean and standard deviation. Paired t test was used to calculate the difference between the means. All the data both in hard copies (questionnaires and consent forms) and soft copy was kept at a central and safe place and was accessible at all times

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TABLE 3.6: TOOLS AND TECHNIQUES FOR DATA GENERATION

Data parameters	Tools and techniques
Reported data	
Family history of NCDs, medical history, supplementation usage, addiction patterns	Semi-structured questionnaire
Physical activity	International physical activity questionnaire (short form), 2005
Quality of life	Quality of life instrument for Indian diabetes patients
Knowledge attitude and practices	Semi-structured questionnaire
Anthropometric data	
Weight	Digital weighing scale
Height, waist circumference, hip circumference	Fiber glass tape
Waist hip ratio, waist stature ratio, abdominal volume index	Calculated
Bio-physical data	
Blood pressure	Sphygmomanometer
Dietary data	
24 hour dietary recall	Semi-structured questionnaire
Biochemical data	
Hepatitis B surface antigen	Solid phase enzyme immuno assay
Hepatitis C antibody	Solid phase enzyme immuno assay
Total lymphocyte count, neutrophils, lymphocyte, monocytes, eosinophils, basophils, total RBC, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, RDW-CV, platelet distribution, platelet distribution width, mean platelet volume	Fully automated bidirectional 6 part analysers (SYSMEX XN-1000)

TABLE 3.6: TOOLS AND TECHNIQUES FOR DATA GENERATION

Data parameters	Tools and techniques
Biochemical data	
Hs-CRP	Fully automated nephelometry – BN – II
Iron, total iron binding capacity, transferrin saturation, ferritin	Fully automated bidirectionally interfaced chemi luminescent immuno assay
Blood urea nitrogen	Kinetic UV assay
Creatinine	Creatinine enzymatic method
Uric acid	Enzymatic colourimetric test
Calcium	Arsenazo III method, end point
Total cholesterol	Chod pod method
HDL-C	Enzyme selective protection method
LDL-C	Homogenous enzymatic colourimetric assay
Triglycerides	Enzymatic colourimetric method
VLDL-C	Derived from serum triglyceride value
TC/HDL	Derived from TC and HDL
LDL/HDL	Derived from LDL and HDL
TG/H	Derived from triglycerides and HDL
Non HDL-C	Calculated
Non HDL-C/HDL-C	Calculated
Atherogenic index of plasma	Derived from log transformation of triglycerides and HDL
Metabolic Syndrome	IDF classification, 2005
Alkaline phosphatase	ALP IFCC liquid (colourimetric assay)
Bilirubin direct, bilirubin total	Diazo method of Pearlman and Lee, endpoint
Bilirubin indirect	Derived from serum total and direct bilirubin values
Gamma glutamyl transferase	IFCC standardised SZASZ method
Aspartame aminotransferase, alanine amino transferase	IFCC without pyridoxal phosphate activation

TABLE 3.6: TOOLS AND TECHNIQUES FOR DATA GENERATION

Data parameters	Tools and techniques
Biochemical data	
Total protein	Biuret method
Albumin	Albumin BCG method (colourimetric assay endpoint)
Tri-iodothyronine, thyroxine	Competitive chemi luminescent immuno assay
Thyroid stimulating hormone	Ultra sensitive sandwich chemi luminescent assay
25 OH Vitamin D	Fully automated chemi luminescent immuno assay
HbA1c	Fully automated HPLC using TOSOH G8
Average blood glucose	Derived from glycosylated hemoglobin
NAFLD Fibrosis Score	Calculated
FIB – 4 Score	Calculated
Imaging data	
Ultrasonography	Sonoline Adara Machine

DETAILED DESCRIPTION OF METHODOLOGIES AND TOOLS USED FOR DATA GENERATION

I. SELF REPORTED DATA

Details of the clinical profile, medical history of NCDs, family history of NCDs, duration of diabetes and drug profile were obtained with the help of a pre-tested semi-structured questionnaire. All the data was obtained by face to face interaction in the language of convenience of the subjects. Other than the drugs consumed, the subjects were also asked to inform about the various health supplements they consumed. Data on addiction patterns such as smoking of cigarettes or chewing of tobacco was also obtained.

Physical activity

The information about the physical activity status of the subjects was elicited with the help of the international physical activity questionnaire, short form. It comprises of a set of four questions that provide information about the vigorous physical activity, moderate physical activity, walking activity and sedentary activity patterns (IPAQ, 2005). The frequency and the duration of each activity were recorded and multiplied with the respective domain of metabolic equivalent (MET) scores; heavy (8), moderate (4) and walking (3.3), respectively. According to the international physical activity manual, the score for sitting idle was not to be tabulated. Hence, to arrive at the METMinutes/week, a summation of the score of vigorous, moderate and walking activity was obtained. Based on the classification for physical activity proposed by the IPAQ based on the METMinutes/week, the subjects were further classified having high (>3000 total METminutes/week), medium (600-3000 total METminutes/week) and low physical activity status (<600 total METminutes/week).

II. ANTHROPOMETRIC DATA

Weight

Weight was measured to the nearest 0.1kg with the help of a research grade digital weighing scale and it was calibrated prior to each measurement. The subjects were

asked to stand erect with minimum clothing and look straight to get an accurate measurement of the weight.

Height

Height was measured to the nearest 0.1cm with the help of the fibre glass tape. All the necessary precautions were taken prior to taking measurement so as to rule out any chances of error.

Body Mass Index (BMI)

Body mass index was obtained by dividing weight in kilograms by the square of height in meters. BMI = Weight (kg) / Height $(m)^2$

The subjects nutritional status was further classified on the basis of BMI as normal (<22.9), overweight (>23) or obese (>25) based on the Asia Pacific classification (WHO, 2004).

Waist Circumference (WC)

Waist circumference was measured at the level of the umbilicus with the help of a fiberglass tape. A WC of 80 cm for women and 90 cm for men was taken as the cut off for abdominal obesity (Misra et al., 2006). The measurement was made at the approximate midpoint between the lower margin of the last palpable rib and the top of the iliac crest (WHO, 2008).

Hip Circumference (HC)

Hip circumference was measured at the point of maximum protruding part of the buttocks with the help of a fibreglass tape.

Waist Hip Ratio (WHR)

Waist hip ratio was derived by diving waist circumference by the hip circumference to estimate the splanchnic fat accumulation.

WHR = Waist circumference (cm) / Hip circumference (cm)

A waist hip ratio of ≥0.90 in males and ≥0.85 in females was considered to diagnose the presence of abdominal obesity (WHO, 2008).

Waist Stature Ratio (WSR)

Waist stature ratio was calculated by diving waist circumference by height.

WSR = Waist circumference (cm) / Height (cm)

The cut off for abdominal obesity was taken as WSR ≥0.55 in males and WSR ≥0.53 females.

Abdominal Volume Index (AVI)

As an indicator of intra-abdominal fat and adipose tissue volumes, AVI was assessed. Abdominal index volume was calculated by using the following formula:

AVI = [2cm $(\text{Waist})^2$ + 0.7 $(\text{Waist hip ratio})^2$ / 1000].

A value of 24.5 litres was taken for the estimation of obesity (Guerrero-Romero and Rodriguez-Moran, 2003).

Total Abdominal Fat (TAF)

Total abdominal fat was calculated using the following predictive equation (Bhardwaj et al., 2011): TAF = -47,657+1384.11*gender+1466.54*BMI+416.1*WC

Intra-Abdominal Adipose Tissue (IAAT)

Intra-abdominal adipose tissue was calculated using the following equation (Bhardwaj et al., 2011): IAAT = −238.7+16.9×age+934.18×gender+578.09×BMI−441.06×HC+434.2×WC

Subcutaneous Abdominal Adipose Tissue (SCAT)

The subcutaneous abdominal adipose tissue was calculated using the following equation (Bhardwaj et al., 2011):

SCAT = −49,376.4 − 17.15 × age + 1016.5 × gender + 783.3 × BMI + 466 × HC

III. BIOPHYSICAL MEASUREMENT

Blood pressure

Blood pressure was recorded with a mercury sphygmomanometer in a state of relaxation by trained investigator. The measurement was done in a seated position, with feet on the floor and arm supported at heart level (ADA, 2014). A systolic blood pressure of <130 mm Hg and a diastolic blood pressure of <85 mm Hg was considered to be normal.

IV. DIETARY DATA

24 Hour Dietary Recall

Standard cups and spoons were made use of to obtain information about the 24 hour past day food consumed. Care was taken not to gather data on the days of fast, feasting and festivals. The subjects were asked to elaborate on all the foods and liquids consumed on the previous day. The nutritive value was calculated from the data provided by the subjects in terms of cooked volume and estimates of raw amounts were made after standardizing the recipes. The data was entered and analysed in DietCal, a tool for dietary assessment and planning developed by Ms. Gurdeep Kaur from the Department of Dietetics, AIIMS, New Delhi.

V. BIOCHEMICAL MEASUREMENTS

An overnight twelve hour fasting venous blood sample (10 ml) of the subjects was taken to assess the biochemical profile, namely; hepatitis B surface antigen, hepatitis C antibody, complete blood count, hs-CRP, iron profile, kidney profile, lipid profile, liver enzymes, thyroid profile, vitamin D and glycated hemoglobin through standard laboratory procedures.

Hepatitis B surface antigen (HbSAg)

Method: ELISA, solid phase enzyme immunoessay

Principle: An immunometric immunoassay technique is used, which involves the simultaneous reaction of HBsAg in the sample with mouse monoclonal anti-HBs antibody coated onto the wells and a horseradish peroxidase (HRP)-labelled mouse monoclonal anti-HBs antibody in the conjugate. Unbound conjugate is removed by washing.

The bound HRP conjugate is measured by a luminescent reaction. A reagent containing luminogenic substrates (a luminol derivative and a peracid salt) and an electron transfer agent is added to the wells. The HRP in the bound conjugate catalyzes the oxidation of the luminol derivative, producing light. The electron transfer agent (a substituted acetanilide) increases the level of light produced and prolongs its emission. The light signals are read by the system. The amount of HRP conjugate bound is indicative of the level of HBsAg present in the sample.

Viral hepatitis is a major public health problem of global importance with an estimated 300 million persistent carriers of hepatitis B virus (HBV) worldwide. Infection with HBV results in a wide spectrum of acute and chronic liver diseases that may lead to cirrhosis and HCC.

Viral hepatitis is a disease of the liver that is caused by a number of well-characterized viruses including HBV. Transmission of HBV occurs by percutaneous exposure to blood products and contaminated instruments, sexual contact and perinatally from HBV-infected mothers to their unborn child.

HBV infection produces an array of unique antigens and antibody responses that, in general, follow distinct serological patterns. Hepatitis B surface antigen (HBsAg), derived from the viral envelope, is the first antigen to appear following infection and

can be detected serologically as an aid in the laboratory diagnosis of acute HBV infection (NHANES 2011-2012).

Reference range:

Negative: <0.90

Equivocal: 0.91 – 1.10

Positive: >1.11

Hepatitis C antibody (HCV)

Method: HCV ELISA Test Kit, solid phase enzyme immunoessay

Principle: Qualitative determination of the human antibody directed against hepatitis C virus (anti-HCV) in human serum or plasma is measured using direct solid-phase enzyme immunoassay. Human serum or plasma is diluted in specimen diluent and incubated on a microwell coated with recombinant HCV antigen. Following a 1-hour incubation, the plate is washed to remove unbound material. A peroxidase-conjugated antibody directed against human IgG is added to each well on the microwell plate. Following a 60-min incubation, the wells are washed again to remove unbound material. A peroxidase-specific chromogenic substrate solution is added to each well. The substrate solution consists of hydrogen peroxide and o-phenylenediamine (OPD) in a citrate buffer. Following a 30-min incubation at 20-25°C, 1 N sulfuric acid is added to stop the enzyme-substrate reaction.

Anti-HCV antibody will bind to the HCV antigen in the microwell. Subsequently, the conjugate binds to that antibody. The reaction of the conjugate with the substrate solution results in the generation of an orange colour. Absence of colour indicates the absence of anti-HCV in the sample. The intensity of the colour generated is measured spectrophotometrically at 492 nm. A cut off value is calculated based upon values obtained from control reagents included with each testing series. Results are expressed as "positive" or "negative" for anti-HCV. Positive specimens are repeated in duplicate according to the same procedure.

Reference range:

Negative: <0.90

Equivocal: 0.91 – 1.10

Positive: >1.11

Complete Blood Count (CBC)

Method: Complete Blood Count with 6-Part Differential Analyser

The Beckman Coulter method of sizing and counting particles uses measurable changes in electrical resistance produced by nonconductive particles suspended in an electrolyte.

A suspension of blood cells passes through a small orifice simultaneously with an electric current. A small opening (aperture) between electrodes is the sensing zone through which suspended particles pass. In the sensing zone, each particle displaces its volume of electrolyte. Beckman Coulter measures the displaced volume as a voltage pulse, the height of each pulse being proportional to the volume of the particle.

The quantity of suspension drawn through the aperture is for an exact reproducible volume. Beckman Coulter counts and sizes individual particles at a rate of several thousand per second. This method is independent of particle shape, colour, and density.

Methodology: The methods used to derive CBC parameters are based on the Beckman Coulter method of counting and sizing, in combination with an automatic diluting and mixing device for sample processing, and a single beam photometer for hemoglobinometry. The WBC differential uses VCS technology. Analysis and classification of WBCs use three simultaneous measurements of individual cell volume (V), high frequency conductivity (C), and laser light scatter (S). The scattergram plots the cells based upon the measurements of these three parameters.

Total Leucocyte Count (TLC)

It is calculated as percentage lymphocytes multiplied by the WBC divided by 100.

Reference range: 4.4 – 11.0 * 10^3/microL

Neutrophils

The percentages of leukocytes from each category are derived from the scatter plot.

Reference range: 40-80%

Lymphocyte

The percentages of leukocytes from each category are derived from the scatter plot.

Reference range: 20-40%

Monocytes

The percentages of leukocytes from each category are derived from the scatter plot.

Reference range: 0 – 10%

Eosinophils

The percentages of leukocytes from each category are derived from the scatter plot.

Reference range: 0 – 6%

Basophils

The percentages of leukocytes from each category are derived from the scatter plot.

Reference range: 0 – 1%

Total RBC

This is the number of erythrocytes measured directly, multiplied by the calibration constant, and expressed as n x 106 cells/Ml.

Reference range: Males: 4.3-5.9, females 3.5-5 * 10^6/microL

Hemoglobin (Hb)

Method: Cyanmethemoglobin methods

Principle: Blood is diluted with a dilute solution of potassium ferricyanide and potassium cyanide at a slightly alkaline pH. The ferricyanide converts the hemoglobin to methemoglobin. The cyanide then reacts with the methemoglobin to form the stable cyanmethemoglobin. The colour intensity is measured in a spectrophotometer at a wavelength of 540 mm. The optical density is proportional to the concentration of hemoglobin.

Reference range: Males 13.3-17.7, females 11.7-15.7g/dl

Hematocrit (PCV)

This is the relative volume of packed erythrocytes to whole blood, computed as:

Hct (%) = RBC × CV/10

Reference range: Males: 39.8-52.2%, females: 34.9-46.9%

Mean Corpuscular Volume (MCV)

This is the average volume of individual erythrocytes derived from the RBC histogram. The system: multiplies the number of RBCs in each channel by the size of the RBCs in that channel, adds the products of each channel between 36 fL and 360 fL, divides that sum by the total number of RBCs between 36 fL and 360 fL, multiplies by a calibration constant and expresses MCV in femtoliters.

Reference range: 76-100fL

Mean Corpuscular Hemoglobin (MCH)

This is the weight of hemoglobin in the average erythrocyte count, computed as:

Hgb/RBC x 10

Reference range: 27-33pg

Mean Corpuscular Hemoglobin Concentration (MCHC)

This is the average weight of hemoglobin in a measured dilution, computed as:

Hgb/Hct x 100

Reference range: 33.4-37g/dl

RDW-CV

RDW represents the size distribution spread of the erythrocyte population derived from the RBC histogram. It is the coefficient of variation (CV), expressed in percent, of the RBC size distribution. It is derived from the RBC histogram.

Reference range: 11.5-14.5%

Platelet count

This is the number of thrombocytes derived from the Plt histogram and multiplied by a calibration constant. This number is expressed as: n x 103 cells/μL

Reference range: 150-400 * 10^3/micro/L

Platelet Distribution Width (PDW)

PDW is a specific marker of activation of coagulation. An increased PDW is an indication for the anisocytosis of platelets. Reference range: 9.6-15.2fL

Mean Platelet Volume (MPV)

MPV is the average volume of individual platelets derived from the Plt histogram. It represents the mean volume of the Plt population under the fitted Plt curve multiplied by a calibration constant, and expressed in femtoliters.

Reference range: 6.5-12fL

High-sensitivity CRP (hs-CRP)

Method: Nephelometry

Principle: This method quantifies C-reactive protein (CRP) by latex-enhanced nephelometry. Particle-enhanced assays are based on the reaction between a soluble analyte and the corresponding antigen or antibody bound to polystyrene particles. For the quantification of CRP, particles consisting of a polystyrene core and a hydrophilic shell are used in order to link anti-CRP antibodies covalently. A dilute solution of test sample is mixed with latex particles coated with mouse monoclonal anti-CRP antibodies. CRP present in the test sample will form an antigen-antibody complex with the latex particles.

Light scattering, measured by a nephelometric procedure after 6 min, is proportional to the concentration of the analyte present in the sample. An automatic blank subtraction is performed. CRP concentrations are calculated by using a calibration curve. Data reduction of the signals is performed by using a storable logit-log function for the calibration curve. These assays are performed on a Behring Nephelometer for quantitative CRP determination.

Elevated values can be found among people with certain chronic inflammatory diseases, i.e. rheumatoid arthritis, juvenile chronic arthritis, ankylosing spondylitis and Crohn's disease; in diagnosis and therapy of infections, and in premature rupture of membranes or prediction of chorioamnionitis; differential diagnosis of pyelophritis versus cystitis, bacterial versus viral infections, necrotizing pancreatitis versus edematous interstitial pancreatitis; and suspected renal allograft rejection.

TABLE 3.7: CLASSIFICATION OF Hs-CRP ACCORDING TO CDC AND AHA, 2003

Risk level	Risk Category
< 1 mg/l	Low
1 – 3 mg/l	Average
> 3 mg/l	High
> 10 mg/l	Very high

Iron profile

Iron

Method: Photometry

Principle: The method used to measure the iron concentration is a timed-endpoint method. In the reaction, iron is released from transferrin by acetic acid and is reduced to the ferrous state by hydroxylamine and thioglycolate. The ferrous ion is immediately complexed with the FerroZine Iron Reagent. The system monitors the change in absorbance at 560 nm at a fixed time interval. This change in absorbance is directly proportional to the concentration of iron in the sample.

Iron (non-heme) measurements are used in the diagnosis and treatment of diseases such as iron deficiency anemia, chronic renal disease, and hemochromatosis.

Reference range: Males 70-180, females 60-180microg/dl

Total Iron Binding Capacity (TIBC)

Method: Total Iron Binding Capacity (TIBC) is done indirectly by the Unsaturated Iron Binding Capacity (UIBC) method.

Principle: A known ferrous iron standard 105 µmol/L (586 µg/dL) incubated with serum at a pH of 7.9 saturates the available binding sites on serum transferrin. The unbound excess iron is then complexed with ferene® to form ferrous ferene, a blue complex, which is measured by the LX system. The UIBC is equal to the total iron added less the excess iron.

This assay uses a ferroin type compound, Ferene. This reagent is a superior iron chelating agent forming a Ferene complex with ferrous iron with a maximum absorbance at 593 nm and a molar absorptivity of 35,500. LX20 uses 600 nm analytic wavelengths. The compound has a 27% higher molar absorption than ferrozine,

absorbs at a longer wavelength, and has the other advantages of ferrozine, namely its solubility and stability (NHANES 2005-06).

Reference range: Males 225-535mirog/dl, females 215-535microg/dl

Transferrin saturation

Transferrin is the plasma iron transport protein that binds iron strongly at physiological pH levels. Transferrin saturation is the ratio of serum iron and total iron binding capacity.

Reference range: 13-45%

Ferritin

Method: Roche E-170 Ferritin "ECLIA"

Principle: The method for measurement of Ferritin on the Roche Elecsys-170 is a sandwich principle with a total duration time of 18 minutes. The 1st incubation uses 10 uL of sample, a ferritin-specific antibody and a labeled ferritin-specific antibody to form a sandwich complex. The 2nd incubation occurs after the addition of microparticles that cause the complex to bind to the solid phase. The reaction mixture is aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed. Application of a voltage to the electrode then induces chemiluminescent emission which is measured by a photomultiplier. Results are determined via a calibration curve.

Elevated ferritin values are also encountered with the following tumours: acute leukemia, Hodgkin's disease and carcinoma of the lung, colon, liver, and prostate. Ferritin determinations have also proved to be of value in liver metastasis. Reasons for the elevated values could be cell necrosis, blocked erythropoiesis or increased synthesis in tumour tissue (NHANES 2009-10).

Reference range: Males 22-322ng/ml, females 10-291ng/ml

Kidney profile

Blood urea nitrogen (BUN)

Method: Photometry, the LX20 modular chemistry (BUNm) is used to quantitatively determine the concentration of blood urea nitrogen in serum or plasma by means of the enzymatic conductivity rate method.

Principle: A precise volume of sample is injected into the urease reagent in a reaction cup containing an electrode that responds to changes in solution conductivity. Electronic circuits determine the rate of increase in conductivity, which is directly proportional to the concentration of urea in the sample.

Urea nitrogen measurements are used in the diagnosis and treatment of certain renal and metabolic diseases in conjunction with creatinine measurements (NHANES 2005-06).

Reference range: 7.9 – 20mg/dl

Creatinine

Method: Photometry, the LX20 modular chemistry side uses the Jaffe rate method (kinetic alkaline picrate) to determine the concentration of creatinine in serum, plasma, or urine.

Principle: A precise volume of sample is introduced into a reaction cup containing an alkaline picrate solution. Absorbance readings are taken at both 520 nm and 560 nm. Creatinine from the sample combines with the reagent to produce a red colour complex. The observed rate measurement at 25.6 seconds after sample introduction has been shown to be a direct measure of the concentration of the creatinine in the sample.

Creatinine measurements are useful in the diagnosis and treatment of renal diseases (NHANES 2001-02).

Reference range: Males 0.6-1.1mg%, females 0.4-0.8mg%

Uric acid

Method: Photometry, the DxC uses a timed endpoint method to measure the concentration of uric acid in serum, plasma or urine.

Principle: Uric acid is oxidized by uricase to produce allatoin and hydrogen peroxide. The hydrogen peroxide reacts with 4-aminoantipyrine (4-AAP) and 3, 5-dichloro-2-hydroxybenzene sulfonate (DCHBS) in a reaction catalyzed by peroxidase to produce a coloured product. The system monitors the change in absorbance at 520 nm at a fixed time interval. The change in absorbance is directly proportional to the concentration of uric acid in the sample.

Uric acid measurements are used in the diagnosis and treatment of numerous renal and metabolic disorders (NHANES 2011-12).

Reference range: Males 3.5-7.2mg/dl, females 2-6-6.0mg/dl

Calcium

Method: Photometry, Arsenazo III method, end point

Principle: The LX20 system uses indirect (or diluted) ISE methodology to measure calcium concentration in serum, plasma, or urine. The system determines calcium concentration by measuring calcium ion activity in solution. When the sample buffer mixture contacts the electrode, calcium ions complex with the ionophore at the electrode surface. Changes in potential develop at the electrode surface as the reaction occurs. These changes in potential are referenced to a sodium reference electrode. The reference signal is used in calculating the analyte concentrations based on the Nernst equation.

Reference range: 8.8-10.6mg/dl

Estimated Glomerular Filtration Rate (EGFR)

The CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration) equation was developed to create a more precise formula to estimate GFR from serum creatinine and other clinical parameters.

The CKD-EPI equation, expressed as a single equation, is:

$GFR = 141 * \min(Scr/\kappa,1)^{\alpha} * \max(Scr/\kappa, 1)^{-1.209} * 0.993^{Age} * 1.018$ [if female] * 1.159 [if black]

Scr is serum creatinine (mg/dL), κ is 0.7 for females and 0.9 for males, α is -0.329 for females and -0.411 for males, min indicates the minimum of Scr/κ or 1, and max indicates the maximum of Scr/κ or 1 (Levey et al., 2009).

Lipid profile

Total cholesterol (TC)

Cholesterol is measured enzymatically in serum or plasma in a series of coupled reactions that hydrolyze cholesteryl esters and oxidize the 3-OH group of cholesterol. One of the reaction byproducts, H_2O_2 is measured quantitatively in a peroxidase

catalyzed reaction that produces a colour. Absorbance is measured at 500 nm. The colour intensity is proportional to cholesterol concentration.

Elevated levels of cholesterol increase the risk for CHD. Cholesterol is measured to help assess the patient's risk status and to follow the progress of patient's treatment to lower serum cholesterol concentrations (NHANES 2003-04).

Reference range: 125-200mg/dl

High Density Lipoprotein Cholesterol (HDL-C)

Low serum concentrations of HDL-cholesterol are associated with increased risk for CHD. Coronary risk increases markedly as the HDL concentration decreases from 40- to 30 mg/dL. A low HDL-cholesterol concentration is considered to be a value below 35 mg/dL, and high HDL, $\geq$60 mg/dL. HDL-cholesterol values are also used in the calculation of LDL-cholesterol (see LDL section below).

Direct HDL method. HDL is measured directly in serum. The basic principle of the method is as follows. The apoB containing lipoproteins in the specimen are reacted with a blocking reagent that renders them non-reactive with the enzymatic cholesterol reagent under conditions of the assay. The apoB containing lipoproteins are thus effectively excluded from the assay and only HDL-chol is detected under the assay conditions.

The reagents are purchased from Roche/Boehringer-Mannheim Diagnostics. The method uses sulfated alpha-cyclodextrin in the presence of Mg^{+2}, which forms complexes with apoB containing lipoproteins, and polyethylene glycol-coupled cholesteryl esterase and cholesterol oxidase for the HDL-cholesterol measurement (NHANES 2003-04).

Reference range: 35-80mg/dl

Low Density Lipoprotein Cholesterol (LDL-C)

Most of the circulating cholesterol is found in three major lipoprotein fractions: very low density lipoproteins (VLDL), LDL and HDL.

[Total chol] = [VLDL-chol] + [LDL-chol] + [HDL-chol]

LDL-cholesterol is calculated from measured values of total cholesterol, triglycerides and HDL-cholesterol according to the relationship:

[LDL-chol] = [total chol] - [HDL-chol] - [TG]/5

where [TG]/5 is an estimate of VLDL-cholesterol and all values are expressed in mg/dL. LDL carries most of the circulating cholesterol in man and when elevated contributes to the development of coronary atherosclerosis. LDL-cholesterol is measured to assess risk for CHD and to follow the progress of patients being treated to lower LDL-cholesterol concentrations (NHANES 2003-04).
Reference range: <100mg/dl

Triglycerides

Triglycerides are measured enzymatically in serum or plasma using a series of coupled reactions in which triglycerides are hydrolyzed to produce glycerol. Glycerol is then oxidized using glycerol oxidase, and H_2O_2, one of the reaction products, is measured as described above for cholesterol. Absorbance is measured at 500 nm.
High levels of serum triglycerides help mark conditions that are associated with increased risk for CHD and peripheral atherosclerosis. High triglycerides are associated with increased risk for CAD in patients with other risk factors, such as low HDL-cholesterol, some patient groups with elevated apolipoprotein B concentrations, and patients with forms of LDL that may be particularly atherogenic (NHANES 2003-04).
Reference range: 25-150mg/dl

Very Low Density Lipoprotein Cholesterol (VLDL-C)

VLDL-C is released by the liver into the blood and are converted to LDL-C when they loose triglycerides.
Reference range: 5-40mg/dl

Non-HDL-C

It was calculated by deducting HDL-C from total cholesterol to determine risk prediction for CHD. It is also known as the summation of VLDL-C and LDL-C. It contains all the lipoproteins that have Apo B, a major atherogenic apolipoprotein. It is taken as a surrogate marker for Apo B (NCEP ATP IV).
Non-HDL-C = Total cholesterol – HDL-C
Reference range: <130mg/dl

TABLE 3.8: NATIONAL CHOLESTEROL EDUCATION PROGRAM (NCEP) ADULT TREATMENT PANEL (ATP) IV CLASSIFICATION FOR HIGH BLOOD CHOLESTEROL

Lipoprotein fraction	Value	Category
Total cholesterol (mg/dl)	< 200	Desirable
	200 – 239	Borderline high
	≥ 240	High
LDL cholesterol (mg/dl)	< 100	Optimal
	100 – 129	Near optimal / above optimal
	130 – 159	Borderline high
	160 – 189	High
	≥ 190	Very high
Triglycerides (mg/dl)	< 150	Normal
	150 – 199	Borderline high
	200 – 499	High
	≥ 500	Very high
HDL-C (mg/dl)	< 40	Low
	≥ 60	High

Lipoprotein Ratios

Since the lipoprotein ratios have a greater predictive power in risk assessment for cardiac disease, than the independent lipoprotein fractions alone (Millan et al., 2009), the ratio of LDL/HDL, TC/HDL and TG/HDL were calculated.

LDL/HDL

It is a ratio of LDL-C by HDL-C and helps in cardiovascular disease risk assessment. A ratio above 3.5 was considered to be a risk factor for adverse cardiac event. It is also a good predictor of the degree of clinical benefit derived from lipid lowering intervention (Criqui and Golam, 1998).

TC/HDL

It is also known as the atherogenic or Castelli index. It is considered to be more sensitive and specific than total cholesterol for assessment of cardiovascular risk (Millan et al., 2009). It has also shown to be a good predictor of carotid intima-media thickness (CIMT); and known to have greater power than the isolated variables and similar power to that of the apoB/apoA-I ratio and non-HDL cholesterol (Frontini et al., 2007). A value > 5 was considered as a risk factor. It is also a good predictor of the degree of clinical benefit derived from lipid lowering intervention (Criqui and Golam, 1998). It is a good predictor of cardiovascular risk irrespective of other factors. It becomes useful in the presence of hypertriglyceridemia (Millan et al., 2009).

NON-HDL/HDL

The non-HDL cholesterol/HDL ratio is a lineal combination of total/HDL cholesterol. Although few studies have evaluated this lipoprotein ratio for predicting cardiovascular disease, and as it is a lineal combination of the results can be assumed to be similar to those of the total/HDL cholesterol or LDL/HDL cholesterol ratios (Rader et al., 2003).

TG/H

The ratio of triglycerides to HDL-cholesterol ratio (TG/HDL-C) correlates inversely with the plasma level of small, dense LDL particles (Hanak et al., 2004). TG/HDL-C ratio >4 is the most powerful independent predictor of CAD development (da Luz et al., 2008). Highly significant independent predictor of myocardial infarction, even stronger than TC/HDL-c and LDL-c/HDL-c (Gaziano et al., 1997). It is an easy, non-invasive means of predicting the presence and extent of coronary atherosclerosis (da Luz et al., 2008).

Atherogenic Index of Plasma (AIP)

AIP is a reflection of the delicate metabolic interactions within the whole lipoprotein complex (Dobiasova, 2004). The logarithmic association reflects the balance between atherogenic and protective lipoproteins and is useful for predicting plasma

atherogenicity (Millan et al., 2009). An AIP <0.11 was considered as low risk, between 0.11 to 0.21 as intermediate risk and above 0.21 as increased risk of cardiovascular risk (Frohlich and Dobiášová, 2003).

Diabetic Dyslipidemia

It was defined as the presence of one or more than one of the following lipid aberrations: total cholesterol, triglycerides, LDL-C and HDL-C.

Metabolic Syndrome

The presence of metabolic syndrome was defined on the basis of the classification proposed by International Diabetes Federation (IDF, 2006). The specific reason for choosing this classification was the inclusion of waist circumference in the criteria for defining metabolic syndrome, as it depicts central obesity. Thus, the presence of metabolic syndrome was defined as abdominal obesity plus two or more of the following risk factors:

- Fasting glucose >100 mg/dl or previously diagnosed type 2 diabetes
- Blood pressure >130/85 mmHg
- Triglycerides >150 mg/dl or on specific treatment for the said condition
- HDL-C <40 mg/dl in case of men and <50 mg/dl in case of women
- Waist circumference ≥90 cm in men and ≥80 cm in women

Liver function test (LFT)

Alkaline phosphatase

In the presence of magnesium ions, *p*-nitrophenylphosphate is hydrolyzed by phosphatases to phosphate and *p*-nitrophenol. The rate of *p*-nitrophenol liberation is proportional to the ALP activity and can be measured photometrically.

Reference range: Males 53-128U/l, females 42-98U/l

Bilirubin total, bilirubin direct, bilirubin indirect

Method: It was estimated with the Diazo method- End Point method.

Principle: Total bilirubin is coupled with diazonium salt DPD (2,5-dichlorophenyldiazonium tetrafluoroborate) in a strongly acidic medium (pH 1-2). The intensity of the colour of the azobilirubin produced is proportional to the total bilirubin concentration and can be measured photometrically. Direct bilirubin or conjugated bilirubin is water soluble and it directly reacts in acidic medium. However, for indirect bilirubin or unconjugated bilirubin a surfactant is used for dissolving it in water and then the dissolved material reacts similar to direct bilirubin.

Bilirubin is an organic compound formed by the reticuloendothelial system during the normal and abnormal destruction of red blood cells. Elevated levels are associated with hemolytic jaundice, paroxysmal hemoglobinuria, pernicious anemia, polycythemia, icterus neonatorum, internal hemorrhage, acute hemolytic anemia, malaria, and septicemia. Low bilirubin levels are associated with aplastic anemia, and certain types of secondary anemia resulting from toxic therapy for carcinoma and chronic nephritis.

Direct (conjugated) bilirubin couples with diazotized sulfanilic acid (p-diazobenzenesulfonic acid), forming a blue colour at alkaline pH.

Direct bilirubin (conjugated) + diazotized sulfanilic acid alkaline pH > blue colour azobilirubin

Indirect (unconjugated) bilirubin is diazotized only in the presence of an "accelerating" agent, caffeine-benzoate-acetate mixture. Thus, the blue azobilirubin produced in mixtures containing "accelerating" agent originates from both the **Direct** and **Indirect** fractions and reflects the **Total** bilirubin concentration.

Total bilirubin + caffeine-benzoate-acetate mixture + diazotized sulfanilic acid 6 azobilirubin

Reference range:

Total bilirubin: 0.3-1.2mg/dl

Direct bilirubin: 0-0.2mg/dl

Indirect bilirubin: 0-0.9mg/dl

Gamma glutamyl transferase (GGT)

Principle: In this rate method, L-γ-glutamyl-3-carboxy-4-nitroanilide is used as a substrate and glycylglycine as a acceptor. The rate at which 5-amino-2-nitrobenzoate

is liberated is proportional to γ-GT activity and is measured by an increase in absorbance.

γ-GT measurement is principally used to diagnose and monitor hepato-biliary disease. It is currently the most sensitive enzymatic indicator of liver disease, with normal values rarely found in the presence of hepatic disease. It is also used as a sensitive screening test for occult alcoholism. Elevated levels are found in patients who chronically take drugs such as phenobarbital and phenytoin (NHANES 2007-08).

Reference range: Males 0-55U/l, females 0-38U/l

Aspartate aminotransferase (AST or SGOT)

α-Ketoglutarate reacts with L-aspartate in the presence of AST to form L-glutamate plus oxaloacetate. The indicator reaction uses the oxaloacetate for a kinetic determination of NADH consumption.

As a group, the transaminases catalyze the interconversion of amino acids and α-keto acids by transferring the amino groups. The enzyme AST has been demonstrated in every animal and human tissue studied. Although the enzyme is most active in the heart muscle, significant activity has also been seen in the brain, liver, gastric mucosa, adipose tissue, skeletal muscle, and kidneys of humans. AST measurements are used in the diagnosis and treatment of certain types of liver and heart disease. AST is present in both the cytoplasm and mitochondria of cells. In cases involving mild tissue injury, the predominant form of serum AST is from the cytoplasm, with smaller amounts from the mitochondria. Severe tissue damage results in more of the mitochondrial enzyme being released. Elevated levels of the transaminases can signal myocardial infarction, hepatic disease, muscular dystrophy, or organ damage.

Reference range: Males 0-37U/l, females 0-31U/l

Alanine amino transferase (ALT or SGPT)

α-Ketoglutarate reacts with L-alanine in the presence of ALT to form L-glutamate plus pyruvate. The pyruvate is used in the indicator reaction for a kinetic determination of the reduced form of nicotinamide adenine dinucleotide (NADH) consumption.

As a group, the transaminases catalyze the interconversion of amino acids and α-keto acids by transferring the amino groups. The enzyme ALT been found to be in highest

concentration in the liver, with decreasing concentrations found in kidney, heart, skeletal muscle, pancreas, spleen, and lung tissue. Alanine aminotransferase measurements are used in the diagnosis and treatment of certain liver diseases (e.g., viral hepatitis and cirrhosis) and heart diseases. Elevated levels of the transaminases can indicate myocardial infarction, hepatic disease, muscular dystrophy, or organ damage. Serum elevations of ALT activity are rarely observed except in parenchymal liver disease, since ALT is a more liver-specific enzyme than asparate aminotransferase (AST) (1).

Reference range: Males 13-40U/l, females 10-28U/l

Total protein

The LX20 uses a timed rate biuret method to measure the concentration of total protein in serum or plasma. Proteins in the sample combine with the reagent producing alkaline copper-protein chelate. The rate change in absorbance is monitored by a detector at 545 nm. The observed rate of chelate formation is directly proportional to the total protein concentration in the sample.

Total protein measurements are used in the diagnosis and treatment of nutritional disorders and diseases involving the liver, kidney or bone marrow.

Reference range: 6.6-8.3g/dl

Albumin

At the reaction pH, the bromcresol purple (BCP) in the Roche Diagnostics (RD) albumin system reagent binds selectively with albumin. Although BCP is structurally similar to the conventional bromcresol green (BCG), its pH colour change interval is higher (5.2–6.8) than the colour change interval for BCG (3.8–5.4), thus reducing the number of weak electrostatic dye/protein interactions. The BCP system eliminates many of the nonspecific reactions with other serum proteins as a result of the increased pH. In addition, the use of a sample blank eliminates background spectral interferences not completely removed by bichromatic analyses.

Albumin constitutes about 60% of the total serum protein in normal, healthy individuals. Unlike most of the other serum proteins, albumin serves a number of functions which include transporting large insoluble organic anions (e.g., long-chain fatty acids and bilirubin), binding toxic heavy metal ions, transporting excess

quantities of poorly soluble hormones (e.g., cortisol, aldosterone, and thyroxine), maintaining serum osmotic pressure, and providing a reserve store of protein. Albumin measurements are used in the diagnosis and treatment of numerous diseases primarily involving the liver or kidneys.

Reference range: 3.5-5.2g/dl

Thyroid function

Tri-iodothyronine (T3)

The Access Free T3 assay is a paramagnetic particle, chemiluminescent immunoassay for the quantitative determination of free triiodothyronine levels in human serum and plasma using the Access Immunoassay Systems. The Free T3 Assay is a competitive binding immunoenzymatic assay. Sample is added to a reaction vessels with an anti-T3 monoclonal antibody conjugated to alkaline phosphatase. During the incubation, free T3 in the sample reacts with the anti-T3 antibody. Particles coated with streptavidin and biotinylated T3 analog are then added to the mixture. Unoccupied binding sites on the anti-T3 antibody are bridged to the particle through the T3 analog. After incubation, materials bound to the solid phase are held in a magnetic field while the unbound materials are washed away. A chemiluminescent substrate, Lumi-Phos 530, is added to the reaction vessel and light generated by the reaction is measured with a luminometer. The light production is inversely proportional to the concentration of Free T3 in the sample. The amount of analyte in the sample is determined by means of a stored calibration curve.

Free T4 and Free T3 regulate normal growth and development by maintaining body temperature and stimulating calorigenesis. Free T4 and Free T3 affect all aspects of carbohydrate metabolism as well as certain areas of lipid and vitamin metabolism.

With normal levels of thyroid binding proteins, free T3 levels correlate with Total T3. Measuring free T3 is useful when altered levels of total T3 occur due to changes in thyroid hormone binding proteins, especially in cases with altered TBG or low albumin concentrations. Free T3 is elevated alone (T3 toxicosis) in about 5% of hyperthyroids (NHANES 2011-12).

Reference range: 60-200ng/dl

Thyroxine (T4)

The Access Free T4 (FRT4) assay is a two-step enzyme immunoassay. Monoclonal anti-Thyroxine (T4) antibody coupled to biotin, sample, buffered protein solution, and streptavidin-coated solid phase are added to the reaction vessel. During this first incubation the anti-T4 antibody coupled to biotin binds to the solid phase and the free T4 in the sample. After incubation in a reaction vessel, materials bound to the solid phase are held in a magnetic field while unbound materials are washed away. Next, buffered protein solution and triiodothyronine (T3)-alkaline phosphatase conjugate are added to the reaction vessel. The T3-alkaline phosphatase conjugate binds to the vacant anti-T4 antibody binding sites. After incubation in a reaction vessel, materials bound to the solid phase are held in a magnetic field while unbound materials are washed away. Then, the chemiluminescent substrate is added to the vessel and light generated by the reaction is measured with a luminometer. The light production is inversely proportional to the concentration of free T4 in the sample. The amount of analyte in the sample is determined from a stored, multi-point calibration curve.

The hormone 3,5,3',5'tetraiodothyronine (L-thyroxine,T4) is the most commonly measured substance in the assessment of thyroid function. Most of the thyroxine secreted into the bloodstream is bound to a transport protein, thyroxine binding globulin (TBG), and to albumin and pre-albumin. The small fraction (less than 0.1%) of T4 not bound to these proteins is believed to be the metabolically active hormone as well as the precursor to the physiologically active T3 concentration. This fraction of total T4 concentration is called free thyroxine (free T4, FT4).

Free thyroxine is used as an indicator of patient thyrometabolic status. Free thyroxine is a better indicator than total T4 in that it is not affected by levels of T4 binding proteins (NHANES 2007-08).
Reference range: 4.5-12microg/dl

Thyroid Stimulating Hormone (TSH)

IMx Ultrasensitive hTSH II is a Microparticle Enzyme Immunoassay (MEIA) for the quantitative determination of human thyroid stimulating hormone (hTSH) in serum or plasma on the IMx analyzer.

The IMx Ultrasensitive hTSH II assay is based on the MEIA technology. The IMx Ultrasensitive hTSH II reagents and sample are added to the reaction cell in the following sequence:

The probe/electrode assembly delivers the sample and anti-hTSH coated microparticles to the incubation well of the reaction cell. The hTSH binds to the anti-hTSH coated micro particles forming an antibody-antigen complex. An aliquot of the reaction mixture containing the antibody-antigen complex bound to the micro particles is transferred to the glass fiber matrix. The micro particles bind irreversibly to the glass fiber matrix. The matrix is washed with the wash buffer to remove unbound materials. The Anti-hTSH: alkaline phosphatase conjugated is dispensed onto the matrix and binds with the antibody-antigen complex. The matrix is washed to remove unbound materials. The substrate, 4-methylumbelliferyl phosphate, is added to the matrix and the fluorescent product is measured by the MEIA optical assembly.

In cases of primary hypothyroidism, T_3 and T_4 levels are low and hTSH levels are significantly elevated. In the case of pituitary dysfunction, either due to intrinsic hypothalamic or pituitary disease; i.e., central hypothyroidism, normal or marginally elevated basal TSH levels are often seen despite significant reduction in T_4 and/or T_3 levels. Secondary hypothyroidism typically results in an impaired hTSH response to TRH, while in tertiary hypothyroidism the hTSH response to TRH may be normal, prolonged or exaggerated. Anomalies do occur, however, which limit the use of TRH response as the sole means of differentiating secondary from tertiary hypothyroidism. Although elevated hTSH levels are nearly always indicative of primary hypothyroidism, some rare clinical situations arise which are the result of an hTSH-secreting pituitary tumor (secondary hyperthyroidism). Such patients would display clinical signs of hyperthyroidism.

Primary hyperthyroidism is associated with high levels of thyroid hormones and depressed or undetectable levels of hTSH. The TRH stimulation test has been used in diagnosis of hyperthyroidism. Hyperthyroid patients show a subnormal response to the TRH test. In addition, large doses of glucocorticoids, somatostatin, dopamine and replacement doses of thyroid hormones reduce or totally blunt the hTSH response to TRH (NHANES 2001-02).

Reference range: 0.3-5.5microIU/ml

Vitamin D

Vitamin D is functionally a hormone, rather than a vitamin, and is one of the most important biological regulators of calcium metabolism, in conjunction with parathyroid hormone and calcitonin. As calciferol enters the circulation, it is metabolized to several forms, the primary one being 25-hydroxycalciferol (25-OH-D). The first step in the metabolism of vitamin D, 25 hydroxylation, occurs mainly in the liver. In humans, only a small amount of 25-OH-D is metabolized in the kidney to other di-hydroxy metabolites. Because 25-OH-D is the predominant circulating form of vitamin D in the normal population, it is considered to be the most reliable index of people's vitamin D status. Vitamin D3 (cholecalciferol) is the naturally occurring form of vitamin D produced in the skin after 7-dehydrocholesterol is exposed to solar UV radiation. Vitamin D2 (ergocalciferol) is produced synthetically by UV irradiation of ergosterol. The two forms differ in the structures of their side chains, but they are metabolized identically and have equivalent biological activities.

The measurement of 25-OH-D (referred to as the vitamin D assay) is becoming increasingly important in the management of patients with various disorders of calcium metabolism associated with rickets, neonatal hypocalcemia, pregnancy, nutritional and renal osteodystrophy, hypoparathyroidism, and postmenopausal osteoporosis.

The Diasorin (formerly Incstar) 25-OH-D assay consists of a two-step procedure. The first procedure involves a rapid extraction of 25-OH-D and other hydroxylated metabolites from serum or plasma with acetonitrile. The treated sample is assayed by using an equilibrium RIA procedure. The RIA method is based on an antibody with specificity to 25-OH-D. The sample, antibody, and tracer are incubated for 90 min at 20-25 oC. Phase separation is accomplished after a 20-minute incubation at 20-25oC with a second antibody-precipitating complex (NHANES 2001-02).

Reference range: 30-100ng/ml

Glycosylated hemoglobin

Hemoglobin subfractions formed by the glycation of the alpha or beta chains of hemoglobin A1 (HbA) are collectively known as glycosylated or glycated hemoglobins. Hemoglobin A1c, the best-defined of these, is formed by the reversible

condensation of the carbonyl group of glucose and the amino group at the N-terminus of the beta chain of hemoglobin A, resulting in a labile aldimine or Schiff base. As the red cell circulates, some of the aldimine undergoes a slow, irreversible conversion (Amadori rearrangement) to a stable ketoamine form (HbA1c). Hemoglobin A1c measurements are used in the clinical management of diabetes to assess the long-term efficacy of diabetic control. The glycated hemoglobin result is a reflection of the mean daily blood glucose concentration and the degree of carbohydrate imbalance over the preceding two to three months.

The analyzer dilutes the whole blood specimen with Hemolysis & Wash Solution, and then injects a small volume of the treated specimen onto the HPLC analytical column. Separation is achieved by utilizing differences in ionic interactions between the cation exchange group on the column resin surface and the hemoglobin components. The hemoglobin fractions (A1c, A1b, F, LA1c, SA1c, A0 and H-Var) are subsequently removed from the column material by step-wise elution using Elution Buffers 1, 2 and 3, each with a differing salt concentration. The separated hemoglobin components pass through the photometer flow cell where the analyzer measures changes in absorbance at 415 nm. The analyzer integrates and reduces the raw data, and then calculates the relative percentages of each hemoglobin fraction. Analysis requires three minutes (NHANES 2007-08).

Reference range: <6%

Average Blood Glucose (ABG)

It is the estimated average glucose derived from the glycated hemoglobin. ABG = 28.7 * HbA1c (%) – 46.7

Reference range: 90-120mg/dl

VI. IMAGING DATA

Abdominal Ultrasonography

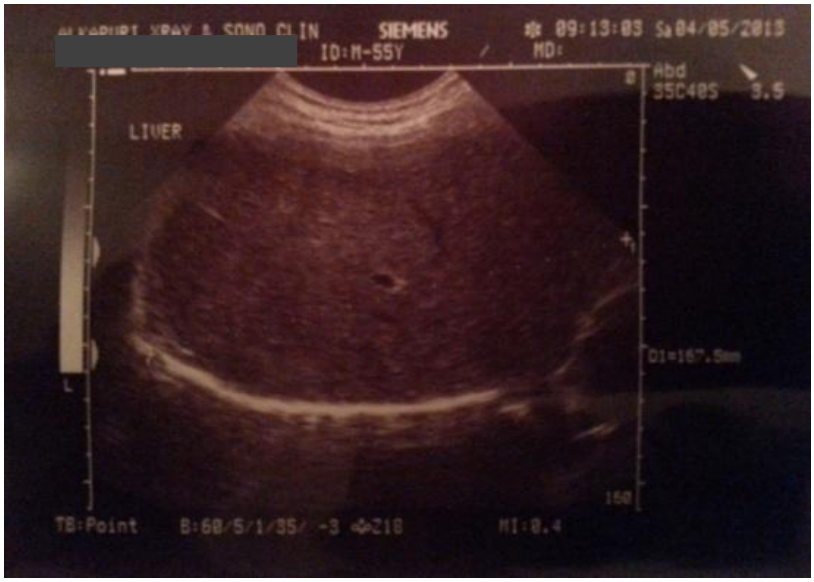
Radiological imaging of the liver with ultrasonography was carried out using Siemens Sono Adara machine with 3.5 MHz transducer by an experienced radiologist who was blinded to the clinical profile of the study subjects. The imaging was done in a supine condition in a state of inspiration with the examiner positioned on the right hand side of the subject. Fatty liver was diagnosed by increased echogenecity of the liver in comparison to the right kidney and the spleen, blurring of intra-hepatic veins and diaphragm and attenuation of the ultrasound beam. The grading of NAFLD into mild, moderate and severe steatosis was based on the visual analysis of the degree of echogenecity by the radiologist (Saadeh et al., 2002).

TABLE 3.9: ULTRASONOGRAPHIC GRADING OF HEPATIC STEATOSIS

Hepatic status	Proportion of liver parenchyma	Description
Normal liver (Grade 0)	< 5 %	Solid homogenous hepatic echo texture, midway between the renal cortex and pancreatic echogenicity
Mild steatosis (Grade 1)	< 33 %	Minimum increase in liver echogenecity with slightly impaired or normal visualization of the diaphragm and intra-hepatic vessels or echogenic liver obscures echogenic walls of portal vein branches
Moderate steatosis (Grade 2)	33 – 66 %	Moderate increase in hepatic echogenecity, definite impaired visualization of diaphragm and intra-hepatic vessels or echogenic liver obscures diaphragmatic outline
Severe steatosis (Grade 3)	> 66 %	Marked increase in hepatic echogenecity, poor or no visualization of the diaphragm and the intra-hepatic vessels

Ref: Mehta et al., 2008

FIG 3.4: ULTRASONOGRAPHIC NORMAL LIVER



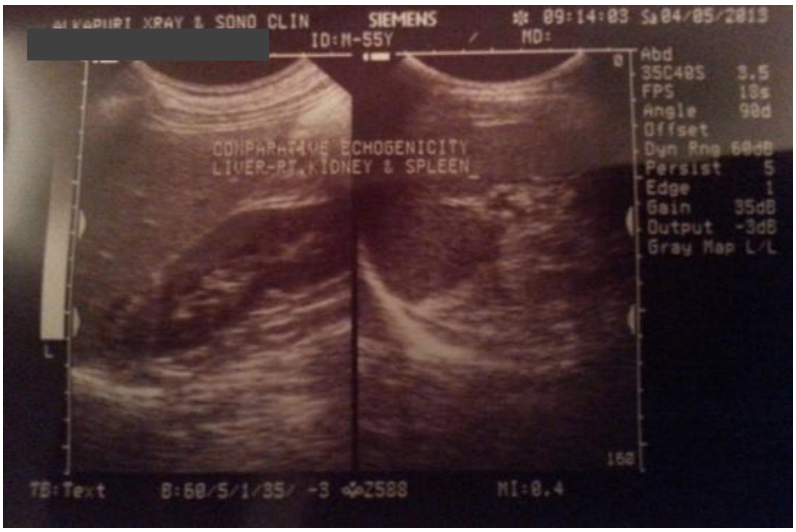


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FIG 3.5: ULTRASONOGRAPHIC GRADE 1 HEPATIC STEATOSIS

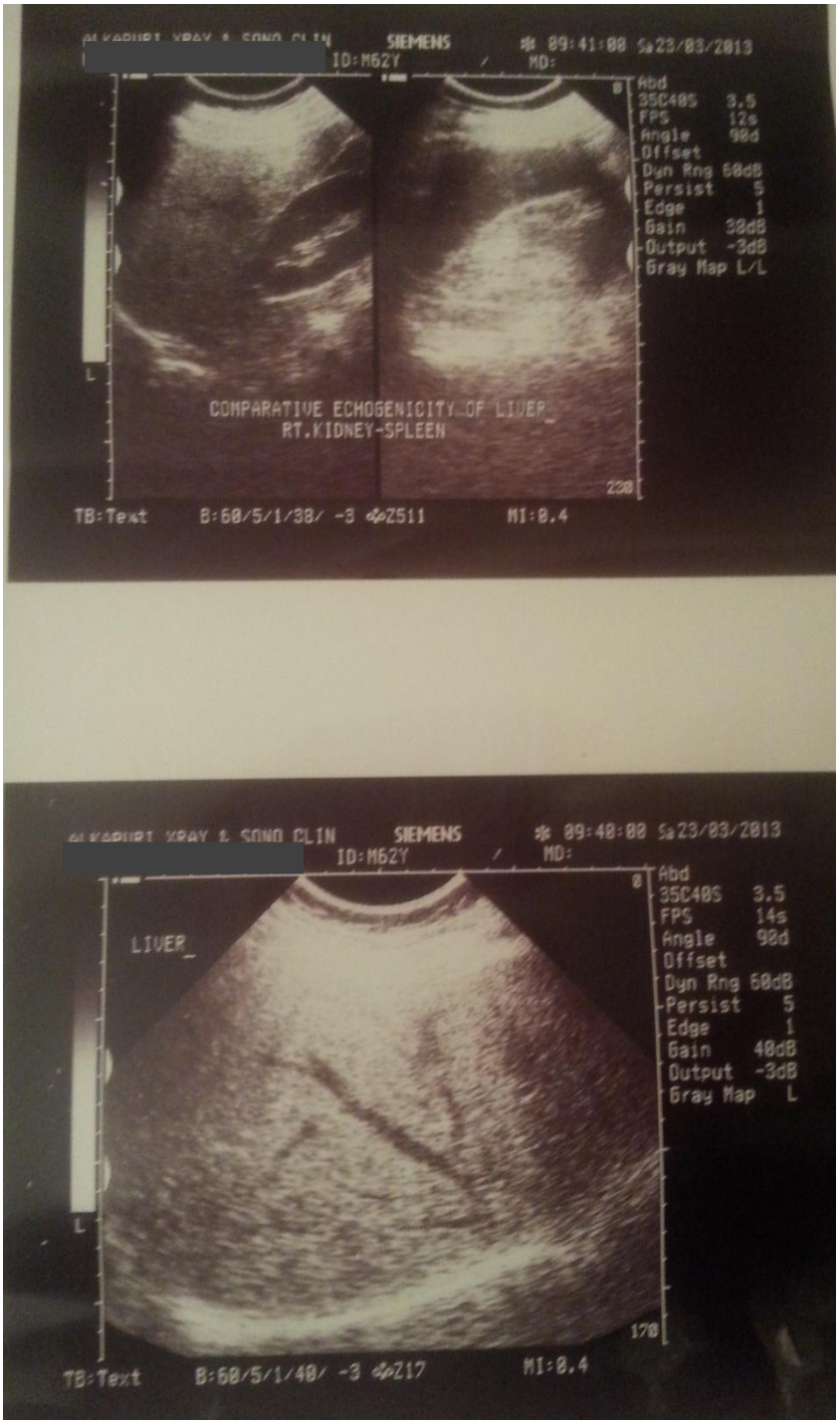


FIG 3.6: ULTRASONOGRAPHIC GRADE 2 HEPATIC STEATOSIS

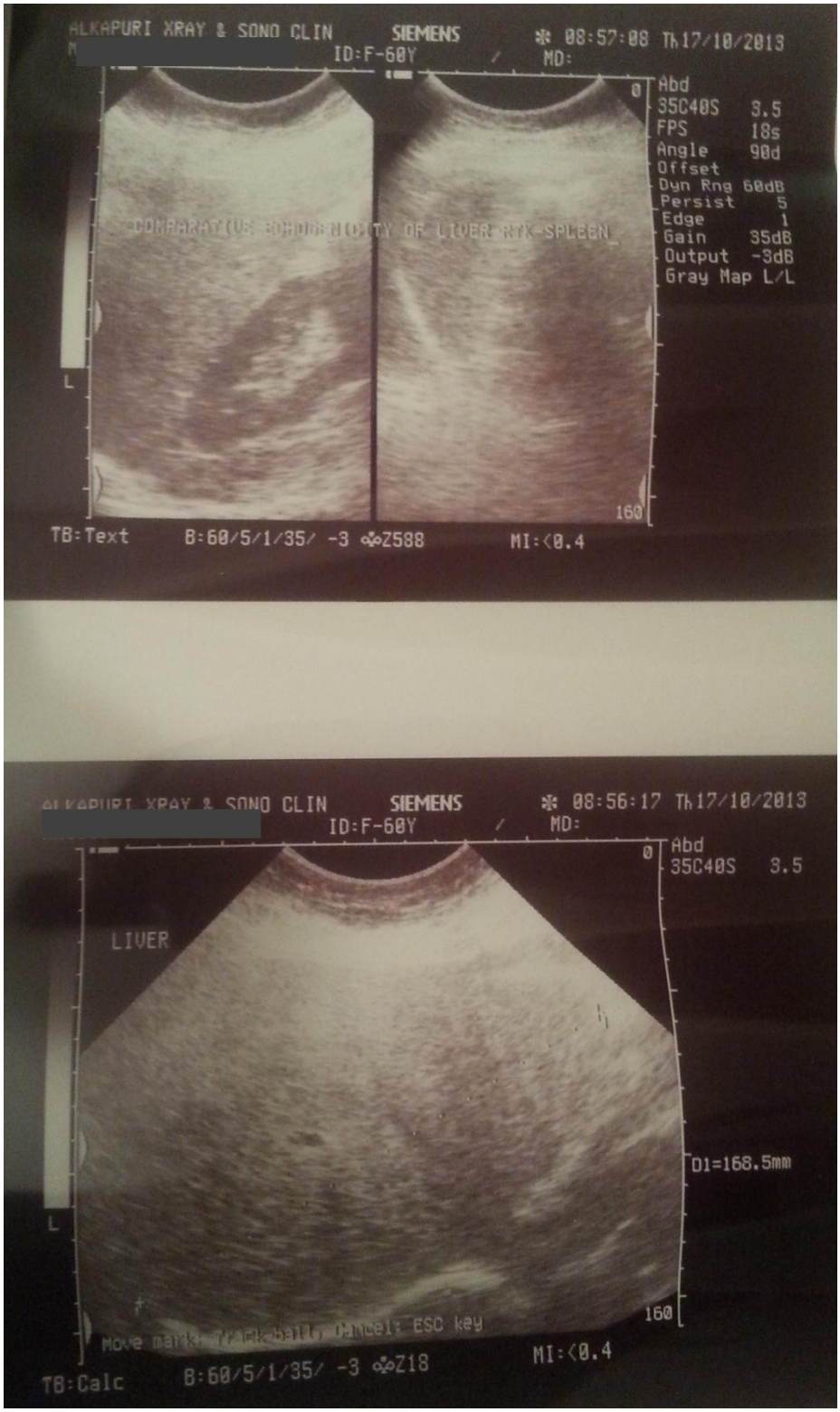
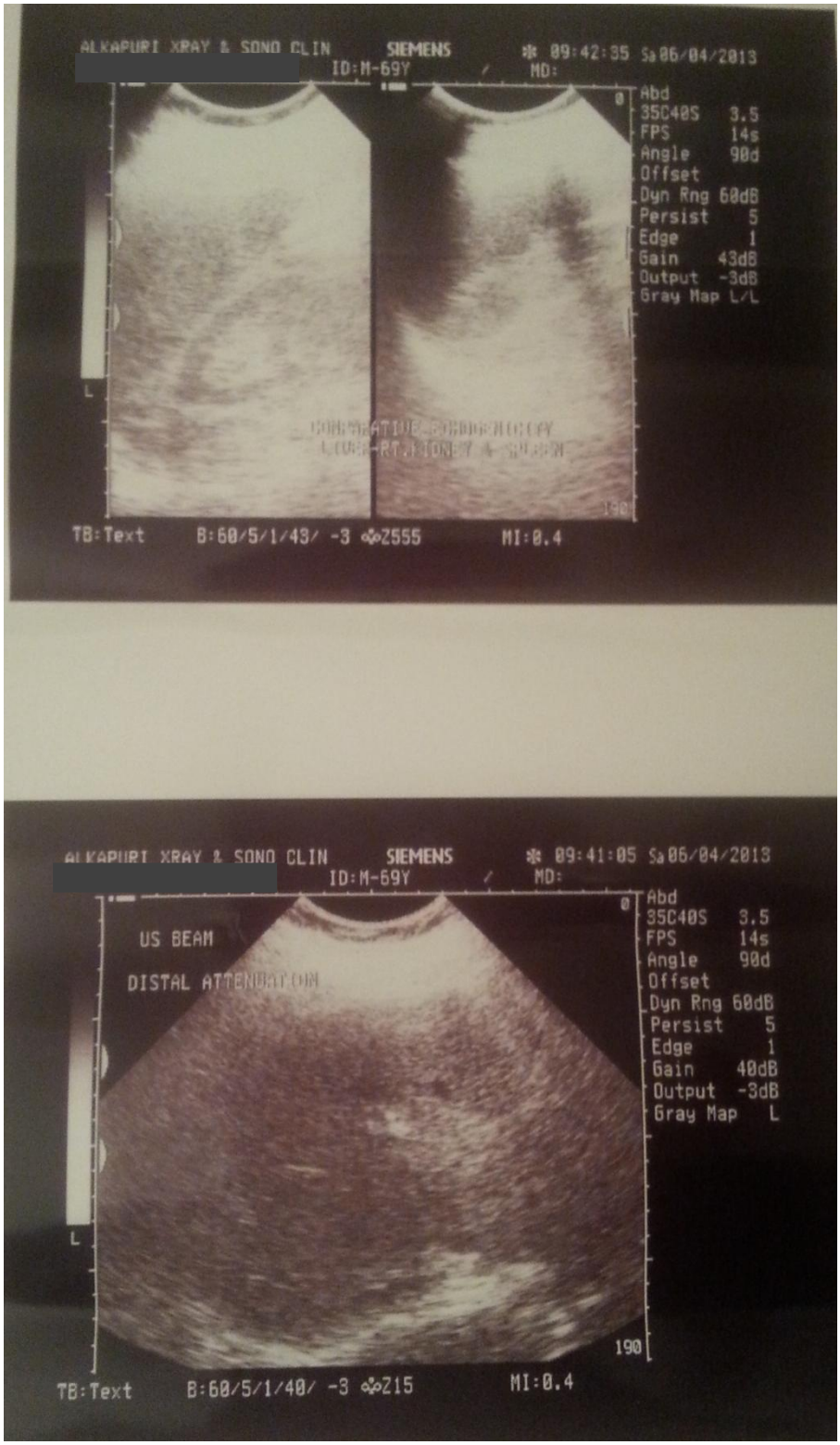


FIG 3.7: ULTRASONOGRAPHIC GRADE 3 HEPATIC STEATOSIS



Liver span

The liver size was estimated by longitudinally scanning the liver in the midclavicular and midline position, by marking the beginning and ending points of hepatic dullness (Kratzer et al., 2003). The distance between these points, derived by ultrasonography, was taken to be the liver size. A liver pan less than 160 mm was considered as normal (Jayarama and Sudha, 2012).

NAFLD Fibrosis Score (NFS)

A simple non-invasive scoring system was developed and validated by Angulo et al. (2007) that can be used to predict the likelihood of presence of bridging fibrosis and/or cirrhosis in patients with NAFLD (Chalasani et al., 2012). It is not used for diagnosing the stage of fibrosis (Noureddin and Loomba, 2012). The scoring system makes use of variables that are commonly evaluated in the routine check ups in a clinical set up, namely; age, body mass index, presence of impaired fasting glucose or confirmed type 2 diabetes, AST, ALT, platelet count and albumin. The score was calculated using the following formula:

NAFLD fibrosis score = -1.675 + 0.037 * age (years) + 0.094 * BMI (kg/m2) + 1.13 IFG/diabetes (yes=1, no=0) + 0.99 * AST/ALT ratio – 0.013 * platelets (*10^9/L) – 0.66 * albumin (g/dL).

A score less than -1.455 depicts less probability of fibrosis; a range of -1.455 to 0.675 indicates probability of intermediate fibrosis and a score >0.675 depicts a very high probability of fibrosis.

FIB – 4

It is a simple non-invasive method to determine liver fibrosis (Sterling et al., 2006 and Vallet-Pichard et al., 2007). It was found to be concordant with the results of FibroTests, the most widely used score to assess fibrosis (Vallet-Pichard et al., 2007). It is easy to calculate and takes into account the routine parameters that are estimated in a clinical set up, namely; age, AST, platelets and ALT. It aids in correct identification of those with moderate and significant fibrosis. It was calculated using the following formula:

FIB – 4 = Age (years) * AST (U/L) / Total platelet count (10^9/L) * √ALT (U/L)

A FIB – 4 score of less than 1.45 depicts early bridging fibrosis, the range of score in the range of 1.45 to 3.25 denotes moderate fibrosis and a score above 3.25 is indicative of significant fibrosis.

STATISTICAL ANALYSIS

The data was entered in Microsoft Excel 2007 and analysed in SPSS 17.0 version (SPSS, Chicago, IL, USA) and Epi Info 3.4.1

1. The quantitative (continuous) variables were presented as mean (student t test) ± standard deviation.
2. Frequencies and percentages were used to derive the quantification of responses of categorical variables.
3. In order to compare the quantitative differences in mean between the three grades of hepatic steatosis, analysis of variance (ANOVA) was performed to obtain F value for continuous variables.
4. A significant F value of ANOVA was further analysed by Bonferroni's test to check for significance between pairs.
5. Pearson correlation was used to define the strength of the association between variables (+1= perfect positive correlation, -1= perfect negative correlation).
6. Forward regression was performed to estimate the numerical relationship between variables.
7. A confidence interval of 95% was taken, which depicted the true population relative risk, based on the sample observations.
8. Bar charts were used to depict frequency distribution for nominal and ordinal data.
9. Line graphs were used to track the chronological change in the quantitative variables over a specific period of time.
10. Chi square test was performed to determine an association between two independent nominal random (categorical) variables.
11. ODDs ratio was calculated to estimate the strength of association between two paired dichotomous random variables.
12. Results were considered to be statistically significant with a 2 tailed p value of <0.05*, P<0.01** and P<0.001***.
13. To assess the impact of intervention, paired t-test was used.