CHAPTER 3

METHODS AND MATERIALS

The study was aimed to assess the effect of vitamin B12 versus calcium with B12 supplementation for eight weeks on neuropathy, quality of life and vitamin B12 status in type 2 diabetes (T2DM) adults on metformin. The study is preliminary of its kind in that it is an attempt to study implications of nutrient - drug interaction. Vitamin B12 absorption is inhibited by metformin and it wad hypothesized that calcium reverses this.

Decrease in vitamin B12 absorption and levels following metformin use typically starts as early as fourth month. Metformin induced B12 deficiency (< 200pg/ml) has been found to be 5.8% to 33% (Yajnik et. al 2006, Pflipsen 2009, Reinstatler et. al 2012) and it is said that this wide range of B12 deficiency is due to varied study definition of B12 deficiency. Calcium reverses the metformin induced low B12 levels in T2DM adults (Bauman et al 2000).

With the above background, the present research work was carried out in three phases as described below:

Phase I: Cross sectional survey of the T2DM adults on metformin

Phase II: Screening for Vitamin B12 status

Phase III: Impact of Vitamin B12 versus Calcium along with Vitamin B12 supplementation

The above mentioned phases were planned with the following **objectives:**

PHASE I: CROSS SECTIONAL SURVEY OF THE T2DM SUBJECTS ON METFORMIN

1. To study the demography, socio economic status, life style and dietary patterns of the T2DM adults on metformin

2. To assess the nutritional status of T2DM adults on metformin by anthropometry using several anthropometric indices.
3. To assess the BP status of T2DM adults on metformin by digital BP apparatus.
4. To assess the status of Diabetes Peripheral Neuropathy (DPN) by Michigan Neuropathy Screening Instrument (MNSI).
5. To assess the Quality of life in T2DM adults on metformin by WHO QoL Bref
6. To conduct market survey for the choice of supplements to be given in Phase III

PHASE II: SCREENING OF T2DM SUBJECTS ON METFORMIN FOR B12 STATUS

1. To study the prevalence of vitamin B12 deficiency among T2DM adults on metformin
2. To study the association of vitamin B12 levels among T2DM adults in relation to DPN and Quality of Life and metformin therapy
3. To assess and study the prevalence of anemia in relation to metformin induced B12 levels

PHASE III: IMPACT OF VITAMIN B12 VERSUS CALCIUM ALONG WITH B12 SUPPLEMENTATION

1. To assess the effect of vitamin B12 versus Calcium with B12 supplementation for eight weeks on vitamin B12 status, neuropathy and quality of life in T2DM adults on metformin.

ETHICAL CLEARANCE: The ethics clearance for the present study was taken from the institutional ethics committee of Foods & Nutrition department, Faculty of Family & Community Sciences, The Maharaja Sayajirao University of Baroda. (No. IECHR/2013/19). Written consent was obtained from all the participants before initiating the data collection (Annexure I). Participants were provided with the participant information sheet explaining the details regarding the study and their participation (Annexure II).

RESEARCH DESIGN

Type of Study: It was a cross sectional mixed longitudinal study.

Locale of the study: It was conducted at Sir Ganga Ram Hospital, Delhi with the support of Dr. Atul Gogia, Senior Consultant, Department of Medicine. (Annexure III)

The entire study was conducted in three phases. Experimental design depicted in Fig. 3.1.

PHASE I: CROSS SECTIONAL SURVEY OF THE T2DM ADULTS ON METFORMIN

A cross sectional survey of previously diagnosed T2DM adults who were coming to the Out Patient Department of Sir Ganga Ram hospital for follow up was done using non-invasive research methods using several non-invasive tools as given in Table 3.1.

A total of 245 T2DM adults were enrolled and data was collected over a period of twenty-six months (December 2013-February 2016).

Based on clinical aspects described by the American Diabetes Association (2013), participants who reported receiving a physician's diagnosis (excluding gestational diabetes) and did not initiate insulin therapy within 1 year of diagnosis were classified as having type 2 diabetes. The subjects were enrolled in the study **only if they were on metformin therapy for a minimum of four months duration.** They were assessed for nutritional status, anthropometry, socio economic status, life style factors, diet history, medical history including drug history, DPN and Quality of Life. The experimental plan of Phase I is given in Figure 3.1.

HOW THE PATIENT WAS ADMINISTERED TO MEET THE STUDY CRITERIA?

In presence of the researcher when there was the first visit of the patient to the OPD of Sir Ganga Ram Hospital, the prescription of patient was searched for: 1st whether the patient was a type 2 diabetes patient, 2nd whether the patient was on metformin, if yes then for how long has he been on metformin. Only when the metformin administration was for a minimum of 4 months the patient was enrolled in the study. Some of the

various brand names of metformin which were looked in the patient prescription included *Diaformin, Glucophage, Riomet, Fortamet, Glumetza, Obimet, Dianben, Diabex, Fortamet, Glucophage XR, Glumetza and others also cross checked by* physician.

Patient's prescriptions and medicines were also searched for prescription of any vitamin B12-containing supplements or calcium supplements and were asked about their use at any time in recent past of 2 months.

SAMPLE SIZE CALCULATION:

Sample size was calculated for sample survey of adults (>20yrs) T2DM on metformin use to estimate the prevalence of low B12 levels in T2DM patients

$N= t^2 \times p(1-p)/m^2$

N=required sample size,

t=confidence level at 95% (1.96)

p= estimated prevalence of low B12 levels in T2DM on metformin = 5.8-33% (based on reviewed literature)

m=margin of error at 5%

$$N = (1.96)^2 \times 0.20(0.8)/ (0.05)^2$$

$$N = 245$$

PHASE II: B12 SCREENING

In phase II all those subjects who gave consent by signing the study consent form (Annexure I) for blood estimations were selected for the study. Out of 245, 167 gave consent for blood estimation but finally 155 participated for blood estimation.

Participants with confounding factors that may affect serum B12 levels were excluded from the study. Keeping this in mind following were the inclusion and exclusion criteria for doing B12 screening:

INCLUSION CRITERIA: Ethnicity: Indo Asians, Serum B12<200 pg/ml, Metformin use of ≥ 4months)

EXCLUSION CRITERIA: High creatinine levels (1.7 mg/dl for men and 1.5 mg/dl for women), hypothyroidism, Alcoholism**,** Pernicious anemia**,** Drugs affecting GI Motility and GI absorption (Proton Pump Inhibitors, H2RAs Hydrogen blockers), Medical history of CRF, liver disease, CKD, Cardiopulmonary disease, Bowel disease/surgery, Cancer, Acid Base disturbance, pregnancy, use of vitamin B12 , multivitamin containing B12 and B12 injections and calcium supplementation. We also excluded pregnant T2DM women and those without diabetes taking metformin.

In all the blood could be collected on 155 T2DM adults and vitamin B12, HbA1c and Hemoglobin with cell morphology were done using C.L.I.A, HPLC and C.B.C respectively on 155 patients.

The biochemical estimations were performed on fasting samples (10-12 h without meal), and 5 ml blood was drawn by a trained technician and analysis was done at an accredited lab of Sir Ganga Ram Hospital, Delhi. The parameters monitored are given in Figure 3.2. The primary outcome was biochemical B12 deficiency determined by serum B12 concentrations. Serum B12 levels were quantified using vitamin B12 Chemiluminescence (C.L.I.A) kit from Sir Ganga Ram hospital laboratories, New Delhi. We defined biochemical B12 deficiency at serum levels ≤200pg/ml (de Benoist, 2008), very low as serum B12 between 149 to 200 pg/ml, and normal as above 200 pg/ml.

In total there were 13 drop outs (12 subjects without blood estimation +1 female subject who was found to have B12<200pg/ml) (Fig 3.1) who did not receive any of the intervention.

Out of 155, a total of 81 T2DM adults were B12 deficient (< 200pg/ml) but one female subject did not come for supplementation due to personal reasons, so in the phase III only 80 subjects participated.

Phase II was followed by Phase III which was an Intervention Trial on T2DM adults on metformin.

PHASE III: INTERVENTION TRIAL (RCT)

Phase III was a Randomized Control Trial (RCT) on T2DM adults on metformin for a minimum of four months duration.

Of 155 subjects screened for B12 status a net of 80 T2DM adults participated in the intervention trial who were randomized into experimental and control group where experimental group received 50 subjects and control group received 30 subjects.

RANDOMIZATION AND ALLOCATION OF TREATMENT

The physician randomly allotted the subjects to control or experimental group by using **'Flipping a Coin'** method for randomization. It is the most common and basic method of simple randomization (Suresh, 2011). In this method the side of the coin received after flipping determines the assignment of each subject to either experimental group or control group. If the flip of a coin produced 'heads' then the subject was assigned to control group however if the flip of a coin produced 'tails' then the subject was assigned to experimental group.

Thirteen subjects did not participate in the intervention trial due to their personal reasons. This drop out resulted in a sample size of 80 subjects in RCT where 50 subjects came in experimental group and 30 in control group at the end of the study.

The experimental group received oral 1000 µg of B12 daily along with oral 500 mg calcium as calcium citrate malate for eight weeks. The control group received oral 1000µg of B12 daily for eight weeks. Standard care remained unaltered during the study period. The experimental group was called as the Ca+B12 group while control group was called as the B12 group.

Serum B12, MNSI scores, glycated hemoglobin (HbA1c) and QoL were assessed before and after intervention. The detailed experimental plan is given in Figure 3.1

RATIONALE FOR THE CHOICE OF THE DESIRED DOSAGES OF SUPPLEMENTATION:

To get an idea of the routine practice prevalent amongst diabetics to correct vitamin B12 deficiency, informal telephonic unstructured interviews were conducted among 10 clinicians (physician, diabetologist and endocrinologist). As per majority of telephonic interviews it was found that no general practice of testing serum B12 among T2DM exists. Some of the clinical practitioners requested B12 test only when they find any clinical complaints in their patients. The B12 supplementation dose given most frequently by them was 1000µg to start with serum B12 levels ≤200 pg/ml. Based on these inputs the supplementation dose of B12 was chosen to be 1000µg/day for those with serumB12 levels ≤200 pg/ml.

As most of the studies have followed B12 levels <200pg/ml as deficient levels, oral supplementation has been planned for T2DM with B12 levels ≤ 200pg/ml at a dose of 1000µg daily for 2 months. Published literature supports that oral and intramuscular supplementation gives similar results at a dose of 1000µg for a week then once every week for four weeks are sufficient to correct vitamin B12 deficiency (Hvas and Nexo 2006; Andres E and Serraj 2012, Chapmana et al 2016)

The calcium supplementation was given in the form of calcium citrate malate at dose of 500mg per day to the experimental group keeping in view the RDA of calcium for Indians.

HOW DATA QUALITY WAS ENSURED?

- The data quality was ensured using valid as well as reliable tools and methods in the study (Table 3.1).
- All biochemical estimations were done using reliable standard procedures usually used at Sir Ganga Ram hospital Labs. Neuropathy assessment was done using valid MNSI tool and Quality of life assessment was done using valid WHO-QOL-BREF.

- The intra rater reliability was ensured for all the anthropometric measurements as well as biophysical measurements by replicating the measurement three times before reporting it in the proforma.
- The interrater reliability was ensured for MNSI while doing neuropathy assessment. The MNSI assessment was replicated by another physician once it has been performed by the researcher on several occasions.

- The researcher was masked to the results of biochemical estimations while performing neuropathy assessment using MNSI tool so as to avoid any kind of researcher's bias.

TABLE 3.1: PARAMETERS AND METHODS FOR DATA COLLECTION

Parameters	**Tools/Method**
Drug history, Medical history, Life Style habits, Anthropometry, Diet history, B.P measurement, Biochemical measurement, Socio Economic Status,	B12 screening proforma (Annexure-IV) (N=245)
Blood Pressure Measurement	Digital Blood Pressure Instrument by Omron(N=245)
Neuropathy scores	MNSI Questionnaire (N=245) (Annexure V)
Assessment of Quality of Life	WHO-QoL Bref (N=245) *(Hindi and English versions)* (Annexure VI and VII)
Serum B12	CLIA (N=155)
Haemoglobin (Hb)	Cyanmethhemoglobin by auto-analyzer (N=155)
C.B.C & cell morphology	C.B.C (N=155)
Glycated Hemoglobin (HbA1c)	HPLC (N=155)

SOCIOECONOMIC STATUS, LIFESTYLE HABITS, DIET AND DRUG HISTORY (ANNEXURE IV):

Socioeconomic status, lifestyle factors, diet and drug history were assessed as mentioned in the B12 screening proforma (Annexure IV).

The socio-economic status was assessed by asking individuals occupation and Per capita income (PCI). PCI was assessed from the individuals by asking various sources of family income per month and then dividing it by total number of family members.

Life style factors like cigarette smoking, tobacco consumption and alcohol consumption were asked from the subjects.

Diet influences the B12 status of individual so the patients were asked as to whether the patient was non-vegetarian or vegetarian or ovo vegetarian. Individuals consuming non-vegetarian foods like meat, fish or chicken at least once weekly were defined as non-vegetarians and those consuming eggs at least twice weekly were defined as ovo vegetarians and those not consuming eggs or non-vegetarian foods but consuming milk were considered vegetarians.

Dietary calcium intake was assessed by asking the major dietary sources of calcium in addition to dairy calcium from milk. The milk consumption was also quantified and individuals consuming less than 200g milk were defined to have low milk intake, those consuming milk in between 200-400g were considered to have moderate milk intake while those consuming more than 400g milk intake were considered to have adequate milk intake.

FIG. 3.1: EXPERIMENTAL DESIGN

PHASE I (CROSS SECTIONAL SURVEY)

(N=245)

Medicine Out Patient Department (OPD), Sir Ganga Ram Hospital, Delhi

T2DM on Metformin use ≥4m

Anthropometric status

BP

DPN

QoL

Medical History

Dietary History

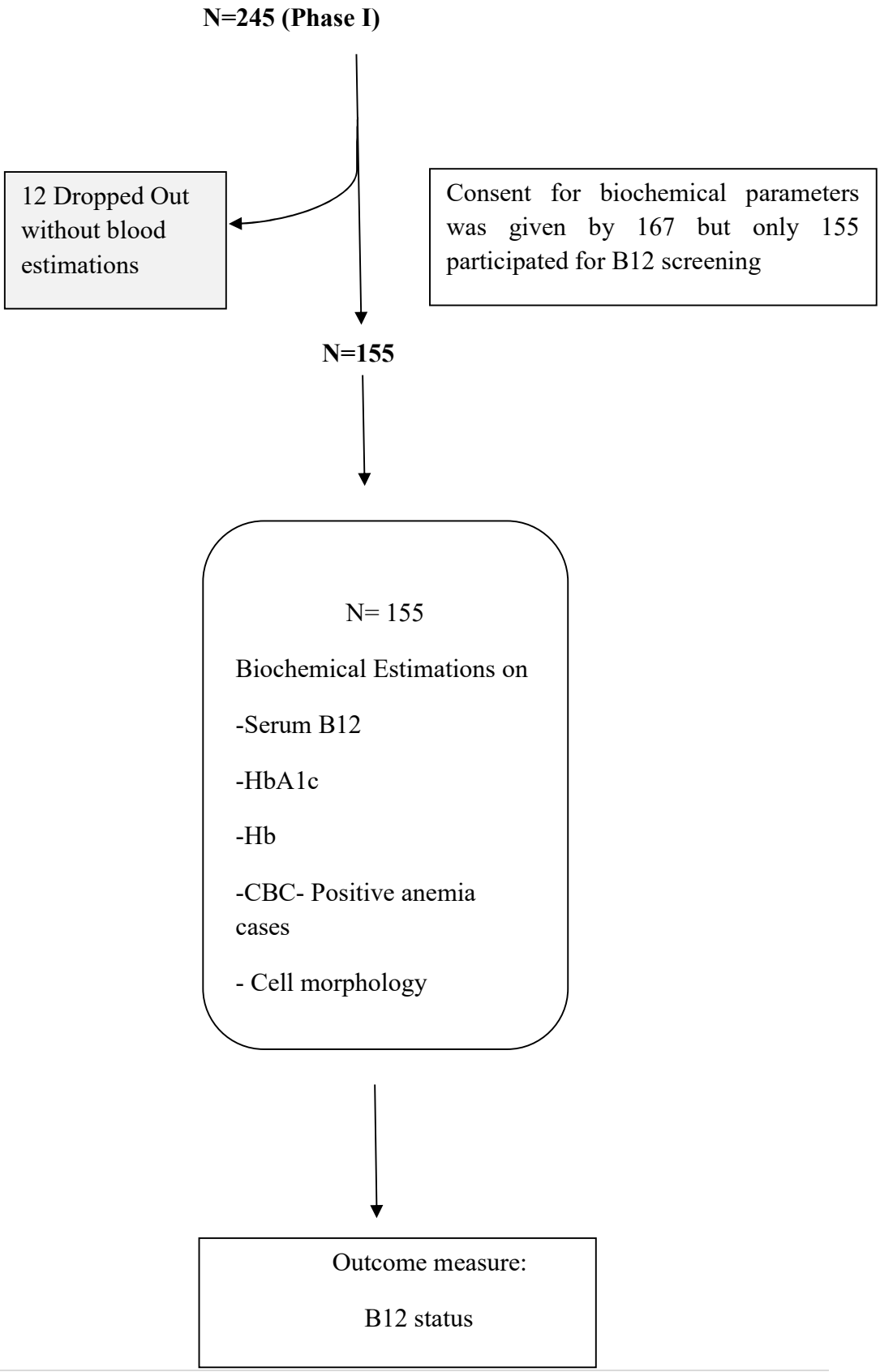
Life Style Habits

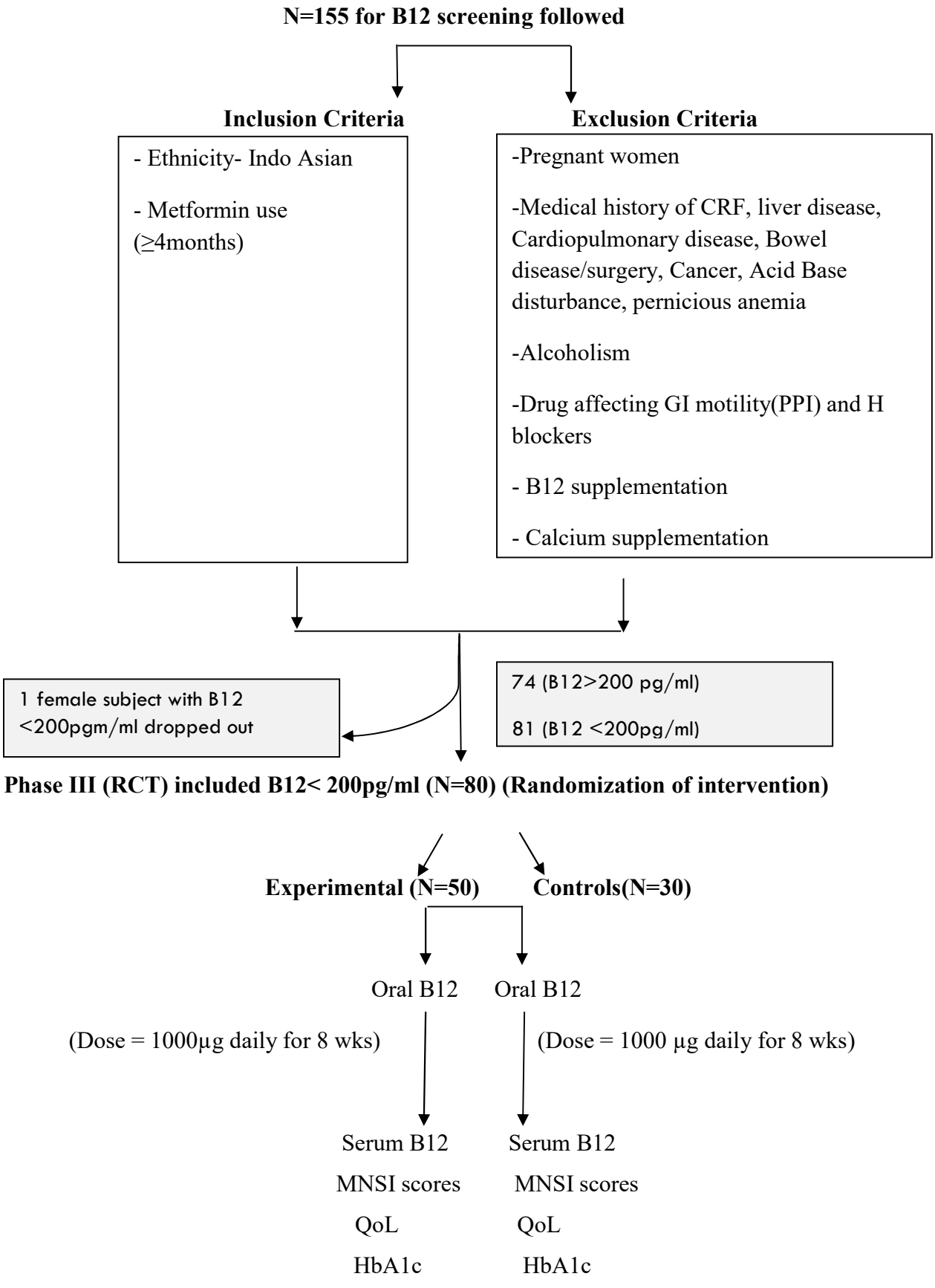
SES

Major outcome measure:

QoL
DPN

PHASE II (SCREENING OF SERUM B12 STATUS)




N=155 for B12 screening followed
Inclusion Criteria
- Ethnicity- Indo Asian
- Metformin use (≥4months)
Exclusion Criteria
-Pregnant women
-Medical history of CRF, liver disease, Cardiopulmonary disease, Bowel disease/surgery, Cancer, Acid Base disturbance, pernicious anemia
-Alcoholism
-Drug affecting GI motility(PPI) and H blockers
- B12 supplementation
- Calcium supplementation
1 female subject with B12 <200pgm/ml dropped out
74 (B12>200 pg/ml)
81 (B12 <200pg/ml)
Phase III (RCT) included B12< 200pg/ml (N=80) (Randomization of intervention)
Experimental (N=50)
Controls(N=30)
Oral B12
(Dose = 1000µg daily for 8 wks)
Oral B12
(Dose = 1000 µg daily for 8 wks)
Serum B12
MNSI scores
QoL
HbA1c
Serum B12
MNSI scores
QoL
HbA1c

ANTHROPOMETRIC MEASUREMENTS

To assess the nutritional status of the subjects, direct parameter of assessment i.e. anthropometric measurements was recorded. The measurements included height, weight, hip and waist circumference. Based on these the body mass index (BMI), waist-to-hip ratio (WHR) and waist-stature ratio (WSR) were calculated. The detailed procedures are discussed in the following section.

- **HEIGHT**

  Height is a linear measurement of the body. It is associated with chronic insufficiency of food intake, frequent infections and continued under nutrition.

  A fiberglass tape was fixed perpendicular to the floor on the wall and the subjects were asked to stand on the floor against the fixed scale with the feet parallel and heels, shoulders and the back of head comfortably erect and not with the knees bent. The subjects were asked to look straight ahead with the head held comfortably erect and the arms hanging loosely by the sides. The head piece of the scale was gently lowered crushing the hair and making the contact with the top of the head. The head piece was at right angles to the scale. The height was measured to the nearest 0.1cm.

- **WEIGHT**

  Weight is a key anthropometric measurement of body mass. When expressed with respect to height, it serves as an index of current nutritional status of and individual. The Salter electronic weighing scale was standardized with the help of standard weight of 5kg and the error was recorded. The subjects were asked to stand on the platform of the scale without touching anything, without foot wear and with minimum clothing. The subjects were asked to look straight ahead without bending down. The equipment was calibrated daily and the minimum weight that the instrument could measure was 0.1 kg.

- **BODY MASS INDEX (BMI)**

Body Mass Index indicates current nutritional status. It is considered as an indicator of weight that is relatively independent on height. BMI is used since it provides an estimate of body fat and is related to risk of disease. Lower BMI thresholds for overweight and obesity have been proposed for the Asia-Pacific region since this population appears to be at risk for glucose and lipid abnormalities at lower body weights. (Powers, 2012).

To calculate BMI, weight and height of the subjects were measured by the standard methods as described above. BMI was calculated using the standard formula:

BMI = Weight (kg)/Height (m^2)

Cut off levels for BMI based on Asia Pacific Classification were used to define overweight and obesity is given in Table 3.2

TABLE 3.2: ASIA PACIFIC CUT-OFFS FOR BODY MASS INDEX (BMI) CLASSIFICATION (WHO 2000)

Classification	BMI (Kg/m^2)
Underweight	<18.5
Normal Weight	18.5 – 22.9
Overweight (at risk)	23 – 24.9
Obese- Grade 1	25 – 29.9
Obese- Grade 2	30 and Above

- **WAIST CIRCUMFERENCE**

Waist circumference is the measure of abdominal obesity and it is well correlated with visceral adipose tissue. The WHO STEPS protocol for measuring instructs that the measurement be made at the approximate midpoint between the lower

margin of the last palpable rib and the top of the iliac crest (WHO 2008, Expert). Excess abdominal fat, assessed by measurement of waist circumference is independently associated with higher risk for DM (Powers, 2012). Measurement of the waist circumference is a surrogate for visceral adipose tissue and should be performed in the horizontal plane above the iliac crest. The midpoint between the inferior margin of the last rib and the crest of ileum in the mid-auxiliary plane was determined. The subjects were asked to stand with their feet together and arms placed on the side with palms of hands facing inwards, and weight evenly distributed across the feet. The subjects were asked to breathe out and a constant tension tape was pressed through the point determined earlier and the measurement was taken to the nearest 0.1cm.

The WHO cut offs used for abdominal obesity states that men with waist circumference ≥90cm (35in) and women with waist circumference ≥80cm (31.5in) are said to have abdominal obesity.

- **HIP CIRCUMFERENCE**

The hip circumference measurement should be taken around the widest portion of the buttocks. The subjects were asked to stand with their feet together, arms placed on sides with palms facing inwards and subjects breathing out. Hip circumference was defined as the widest circumference over the buttocks and below the iliac crest. A non-stretchable fiber glass tape with constant tension was passed through the hips and the measurement was recorded to nearest 0.1 cm.

- **WAIST HIP RATIO (WHR)**

Waist Hip Ratio is one of the widely used measures for abdominal obesity. Increased WHR may reflect both a relative abundance of abdominal fat as assessed by increased waist circumference and relative risk of gluteal muscle

(decreased hip girth). Excess abdominal fat, assessed by measurement of waist hip ratio is independently associated with higher risk for DM (Powers, 2012). WHR is a good indicator for distinguishing gynoid obesity (pear shaped) from android (apple shaped) obesity. The cut offs for WHR states that the individual has abdominal obesity if men has a WHR ≥ 0.90 cm or if women has WHR ≥0.85

- **WAIST STATURE RATIO (WSR)**

WSR, also called Waist to height ratio (WHtR) was calculated by dividing the waist circumference by height in same units. Abdominal obesity was defined as WSR≥0.55 for males and WSR≥0.53 for females.

BLOOD PRESSURE

The blood pressure of all the subjects enrolled in the study was measured by the physician using an automatic blood pressure monitoring machine. The instrument uses the oscillometric method of blood pressure measurement. Three readings at an interval of five minutes each were recorded and a mean value was recorded as the final reading. The cut- offs given by JNC VIII classification were used for assessing the prevalence of hypertension among the subjects. The classification for blood pressure by JNC VIII is given in Table 3.3

TABLE 3.3: JNC VIII CUT-OFFS FOR BLOOD PRESSURE

Classification	Systolic BP (mmHg)	Diastolic BP (mmHg)
Normal	< 120	<80
Pre Hypertensive	120-139	80-90
Stage 1 Hypertension	140-159	90-99
Stage II Hypertension	≥160	≥100

ANALYTICAL PROCEDURES FOR BIOCHEMICAL ESTIMATIONS

The biochemical parameters were performed in the laboratory of Sir Ganga Ram hospital at New Delhi. This lab is certified by NABL (National Accreditation Board for Testing and Calibration Labs, India).

All the methods used for performing biochemical estimations with their detailed procedures are mentioned below:

VITAMIN B12 ESTIMATION

Vitamin B12 is the name given to any one of a group of substances termed cobalamins. They are composed of a tetrapyrrole ring surrounding a central cobalt atom with the side group attached to the cobalt atom. The predominant form in serum is methylcobalamine while the predominant cellular for is 5'deoxyadenosylcobalamine (Nelson and Davey 1991). Cyanocobalamine (MW 1355) is the most stable and is used as a reference compound for measuring serum cobalamin concentrations.

Cobalamins are obtained from animal products such as meat eggs, milk and other dairy products. When ingested they are bound by a protein termed intrinsic factor in the gastric juice of the stomach and are subsequently absorbed in the ileum. Intrinsic factor is required for absorption. Once in circulation, cobalamins are taken up and stored in liver. They are released into the plasma as needed where they are carried by B12 binding proteins (transcobalamins) (Nelson and Davey 1991)

Vitamin B12 is a coenzyme involved in the two very important metabolic functions vital to cell growth and DNA synthesis:

- ✓ The synthesis of methionine
- ✓ The conversion of methylmalonyl CoA to succcinyl Co A

Deficiency of this vitamin can lead to megaloblastic anemia and ultimately to serious neurological problems (Chanarin, I. 1987, Hoffbrand, A.V. 1982). Megaloblastic anemia

is characterized by cell enlargement and reduction in number of all rapidly proliferating cells of the body which is the result of decreased capacity of DNA synthesis.

PRINCIPLE: The principle involved in estimation of serum vitamin B12 is Chemi-luminescence Immuno Assay (CLIA). The Access Vitamin B12 assay is paramagnetic particle, chemiluminescent immunoassay for the quantitative determination of vitamin B12 levels in human serum. CLIA kits are widely used detection method which combine highly sensitive chemiluminescence assay with highly specific immune response and affinity of antibodies.

BLOOD SPECIMEN PREPARATION:

Steps followed while handling, processing and storing blood samples (National committee for Clinical standards 1999):

1. Blood samples were collected by observing all routine precautions for veinpuncture
2. Samples were allowed to clot completely before centrifugation.
3. Tubes were kept stoppered at all the time.
4. Samples were centrifuged at 3500 RPM for 10 mins to remove particulate matter and Fibrin.
5. Within two hours after centrifugation, at least 500µl of cell-free sample was transferred to a storage tube which was stoppered immediately.
6. It was taken care of that the stored samples were not kept at room temperature (15ºC to 30ºC) for more than eight hours.
7. If it was anticipated that the assay would not be completed within eight hours, then the samples were refrigerated at 2ºC to 8ºC.
8. If it was anticipated that the assay would not complete within 24hrs then the samples were frozen at -20ºC or colder. These samples were thawed only once before analysis.
9. Hemolyzed samples were discarded.

ASSAY PROCEDURE

The assay used for B12 estimation is Beckman Coulter Access 2 Immuno Assay system. The Access Vitamin B12 assay is a competitive binding immunoenzymatic assay. A sample was added to a reaction vessel along with alkaline potassium cyanide and dithiothreitol. This treatment denatures B12 binding proteins and convert all forms of B12 to cyanocobalamin form. After neutralization, intrinsic factor alkaline phosphatase conjugate and paramagnetic particles coated with goat anti-mouse IgG: monoclonal anti-intrinsic factors are added to the sample. Vitamin B12 in the sample binds to the intrinsic factor conjugate, preventing the conjugate from binding to the solid phase anti-intrinsic factor.

After incubation in a reaction vessel, materials bound to the solid phase are held in the magnetic field while unbound materials are washed away. Then the chemiluminescent substrate is added to the vessel and light generated is measured with a luminometer. The light production is inversely proportional to the concentration of vitamin B12 in the blood sample. The amount of analyte in the sample is determined from a stored, multi-point calibration curve.

HAEMOGLOBIN ESTIMATION

PRINCIPLE: Blood haemoglobin was estimated using cyanmethhemoglobin method (Drabkin's method) in autoanalyzer. When blood is diluted in Drabkin's solution (consists of potassium cyanide and potassium ferricyanide) it hemolyzes the erythrocytes which release haemoglobin in solution. The ferrous ions Fe2+ of the heamoglobin molecules are oxidized by potassium ferricyanide to ferric ions (Fe3+). Due to this oxidation potassium ferricyanide converts haemoglobin to methhaemoglobin. Further potassium cyanide converts methhaemoglobin to cyanmethhaemoglobin. When measured spectrophotometrically at 540nm, the absorbance of cyanmethhaemoglobin follows Lambert-Beer's Law and is directly proportional to the concentration of haemoglobin in blood. The haemoglobin cut-offs used for diagnosis of anemia as described by WHO (1968) states that anemia is diagnosed if haemoglobin levels < 12.0 g/dl in women and < 13g/dl in men and grades of anaemia are depicted in Table 3.4.

TABLE 3.4: CUT OFFS USED FOR Hb (g/dl) FOR ANAEMIA

Classification	Male	Female
Normal	≥13	≥ 12
Mild	10-12.9	10-11.9
Moderate	8-10	8-10
Severe	< 8	< 8

GLYCATED HAEMOGLOBIN (HbA1c) ESTIMATION

PRINCIPLE: Glycated haemoglobin was estimated using High Performance Liquid Chromatography (HPLC). It is considered a "Gold Standard" technology in estimating the long term plasma glucose of people with DM through the measurement of HbA1c.

HPLC method was used for the percent determination of HbA1c in human whole blood using Variant II Turbo instrument.

It is a chromatographic separation of HbA1c on a cation exchange cartridge. HPLC utilizes a column that holds chromatographic packing material (stationary phase), a pump that moves the mobile phase (s) through the column and a detector that shows the retention times of the molecules. Retention time varies depending on the interaction between the stationary phase, the molecules being analyzed and the solvent(s) used. Cation exchange columns employ the differences in ionic interactions between haemoglobin components to separate them in a span of 1.6 mins. A step gradient elution is used to separate HbF, s-Alc, Total Al, and Hb variant with three types of G8 variant elution buffer His (G8 variant elution buffer His no. 1,2, and 3(s) of different salt concentrations.

TABLE 3.5: CUT OFFS USED FOR HbA1c

Classification	HbA1c
Normal	< 6%
Good control	6-7
Fair Control	7-8
Unsatisfactory	8-10
Poor Control	>10

ASSESSMENT OF DPN

DPN was assessed by Michigan Neuropathy Screening Instrument (MNSI) (See Annexure V). The MNSI consist of two parts, one is a history questionnaire and the other is the physical assessment of lower extremities which includes inspection and assessment of vibratory sensation and ankle reflexes.

1. HISTORY QUESTIONNAIRE:

It consists of fifteen questions about the feelings in legs and feet which are supposed to be completed by the patient but in this study it was interview assisted rather than just being self-administered.

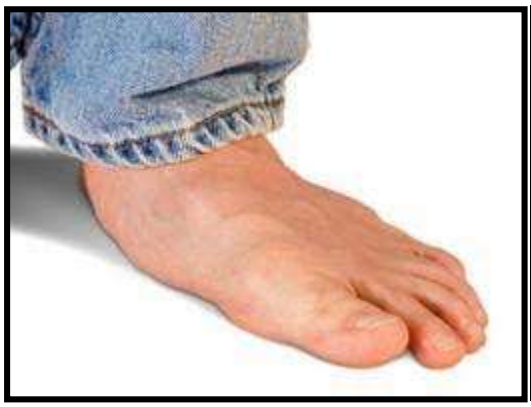
The questions from the history questionnaire was interviewed by the researcher. Responses were added to obtain the total score. Responses of "yes" to items 1-3, 5-6, 8-9, 11-12, 14-15 were each counted as one point. A "no" response on items 7 and 13 counts as 1 point. Item no. 4 was a measure of impaired circulation and item no. 10 was a measure of general aesthenia and these were not included in scoring. To decrease the potential for bias, all scoring information has been eliminated from the patient version.

2. PHYSICAL ASSESSMENT

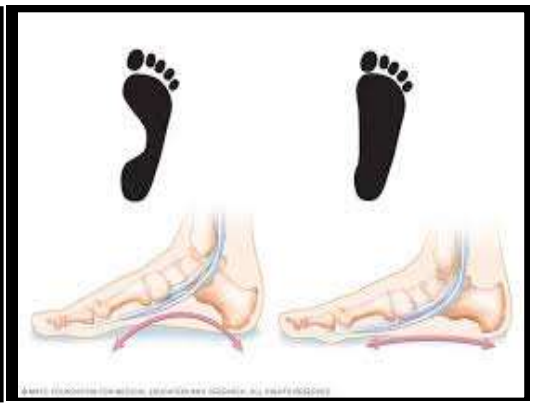
For all assessments, it was required that the foot should be warm (>30°C).

FOOT INSPECTION: The feet were inspected for evidence of excessively dry skin, callous formation, fissures, frank ulceration or deformities. Deformities included flat feet (Fig 3.2), hammer toes (Fig 3.3), overlapping toes (Fig 3.4), hallus valgus (Fig 3.5) (Carl Hueterto defined Hallus Valgus as the most commonly encountered deformity of the forefoot characterized by lateral deviation of the great toe and medial deviation of the first metatarsal with static subluxation of metatarsophalangeal joint. Hallus Valgus is commonly called Bunion), joint subluxation, prominent metatarsal heads, medial convexity known as Charcot foot (Fig. 3.6). Charcot foot, is a condition affecting the bones, joints, and soft tissues of the foot and ankle, characterized by inflammation in the earliest phase. The Charcot foot has been documented to occur as a consequence of various peripheral neuropathies; however, diabetic neuropathy has become the most common etiology (Rogers et al 2011).

FIG 3.2: FLAT FEET

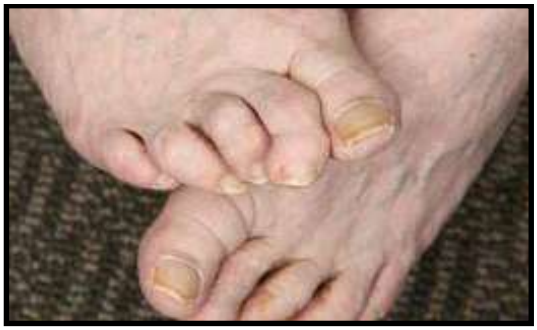


(www.medicalnews.com)



(www.mayoclinic.org)

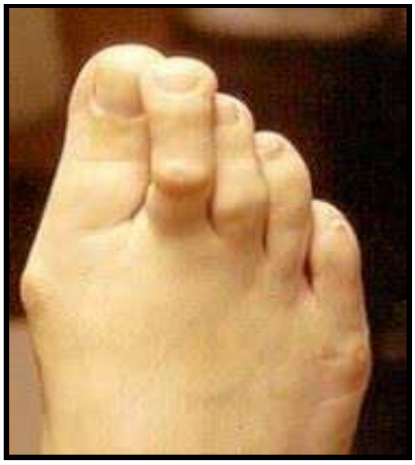
FIG.3.3: HAMMER TOES



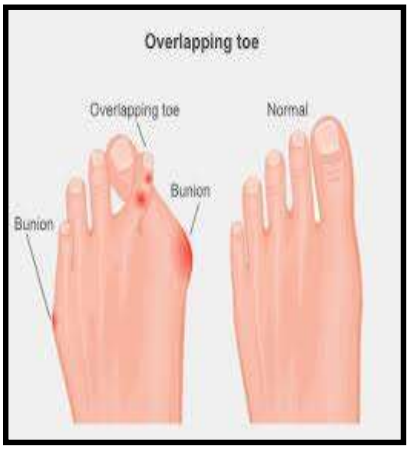
(www.medicalnews.com)

(www.podiatrygroupofgeorgia.com)

FIG. 3.4: OVERLAPPING TOES AND BUNION

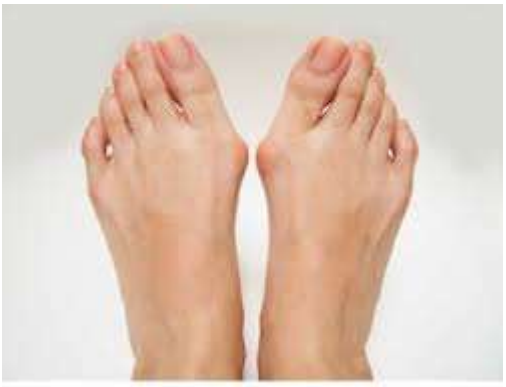


(www.podiatrygroupofgeorgia.com)

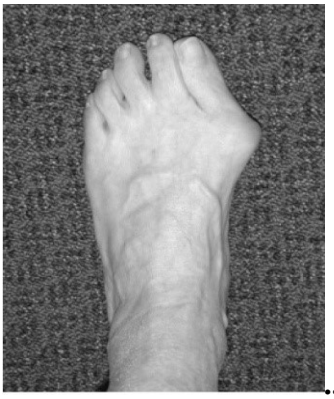


(www.healthinfo.org.nz)

FIG. 3.5: HALLUS VALGUS

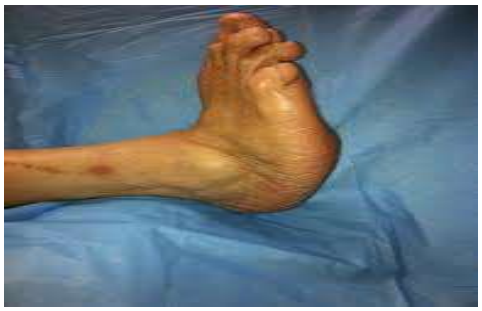


(www.shutterstock.com)



(www.bofas.org.uk)

FIG. 3.6: CHARCOT FOOT



(www.care.diabetesjournal.org)

Each foot with any abnormality received a score of 1. Each foot was also inspected for ulcers and each foot ulcers also received a score of 1.

VIBRATION SENSATION: Vibration sensation should be performed with the great toe unsupported. Vibration sensation will be tested bilaterally using a *128 Hz tuning fork* (Fig.3.7) placed over the dorsum of the great toe on the boney prominence of the DIP joint (Fig. 3.10). Patients, whose eyes were closed were asked to indicate when they can no longer sense the vibration from the vibrating tuning fork.

In general, the examiner should be able to feel vibration from the hand-held tuning fork for 5 seconds longer on his distal forefinger than a normal subject can at the great toe (e.g. examiner's DIP joint of the first finger versus patient's toe). If the examiner feels vibration for 10 or more seconds on his or her finger, then vibration was considered decreased. A trial was given when the tuning fork was not vibrating so as to be certain that the patient is responding to vibration and not pressure or some other clue.

Vibration was scored as:

1) Present if the examiner senses the vibration on his or her finger for < 10 seconds,

2) Reduced if sensed for ≥ 10 or

3) absent (if no vibration was detected by patient)

ANKLE REFLEX/ MUSCLE STRETCH REFLEXES: The ankle reflexes were examined using an appropriate *reflex hammer (e.g. Trommer or Queen square) (Fig.3.7)*. The ankle reflexes were elicited in the sitting position with the foot dependent and the patient relaxed (Fig 3.9). For the reflex, the foot was passively positioned and the foot dorsiflexed slightly to obtain optimal stretch of the muscle. The Achilles tendon was to be percussed directly. If the reflex was obtained, it was graded as present. If the reflex was absent, the patient was asked to perform the *Jendrassic maneuver* (i.e., hooking the fingers together and pulling) (Fig.3.8). Reflexes elicited with the Jendrassic maneuver alone were designated "present with reinforcement." If the reflex was absent, even in the face of the Jendrassic maneuver, the reflex was considered absent.

MONOFILAMENT TESTING: Monofilament testing was done using 10g/5.07 monofilament. (Fig. 3.7). It was bought from the Diabetic Foot Care India Pvt. Limited company (www.diabeticfootcareindia.com).

For this examination, it was important that the patient's foot be supported (i.e., allow the sole of the foot to rest on a flat, warm surface) (fig. 3.11). The filament was initially pre stressed (4-6 perpendicular applications to the dorsum of the examiner's first finger). The filament was then applied to the dorsum of the great toe midway between the nail fold and the DIP joint. Precaution was taken not to hold the toe directly. The filament was applied perpendicularly and briefly, (<1 second) with an even pressure. When the filament bends, the force of 10 grams had been applied. The patient, whose eyes were closed, were asked to respond yes if he/she feels the filament. Eight correct responses out of 10 applications were considered normal: one to seven correct responses indicated reduced sensation and no correct answers were translated into absent sensation.

Precautions taken while using monofilament were:

a. Monofilament was not used on the wound area
b. Monofilament was not bend too much to avoid inappropriate results
c. Monofilament may not produce good results if used for more than 50 bends so it was taken care to replace it with a new one after 20 patients.
d. The length of monofilament was not cut at any point so as to avoid any increase in the force in order to avoid any injury to skin.

FIG 3.7: TOOLS USED FOR MNSI: HAMMER (TOP), 128 Hz TUNNING FORK (MIDDLE), 10g MONOFILAMENT (BOTTOM)

FIG 3.8: JENDRASSIC MANEUVER PERFORMED BY THE SUBJECT

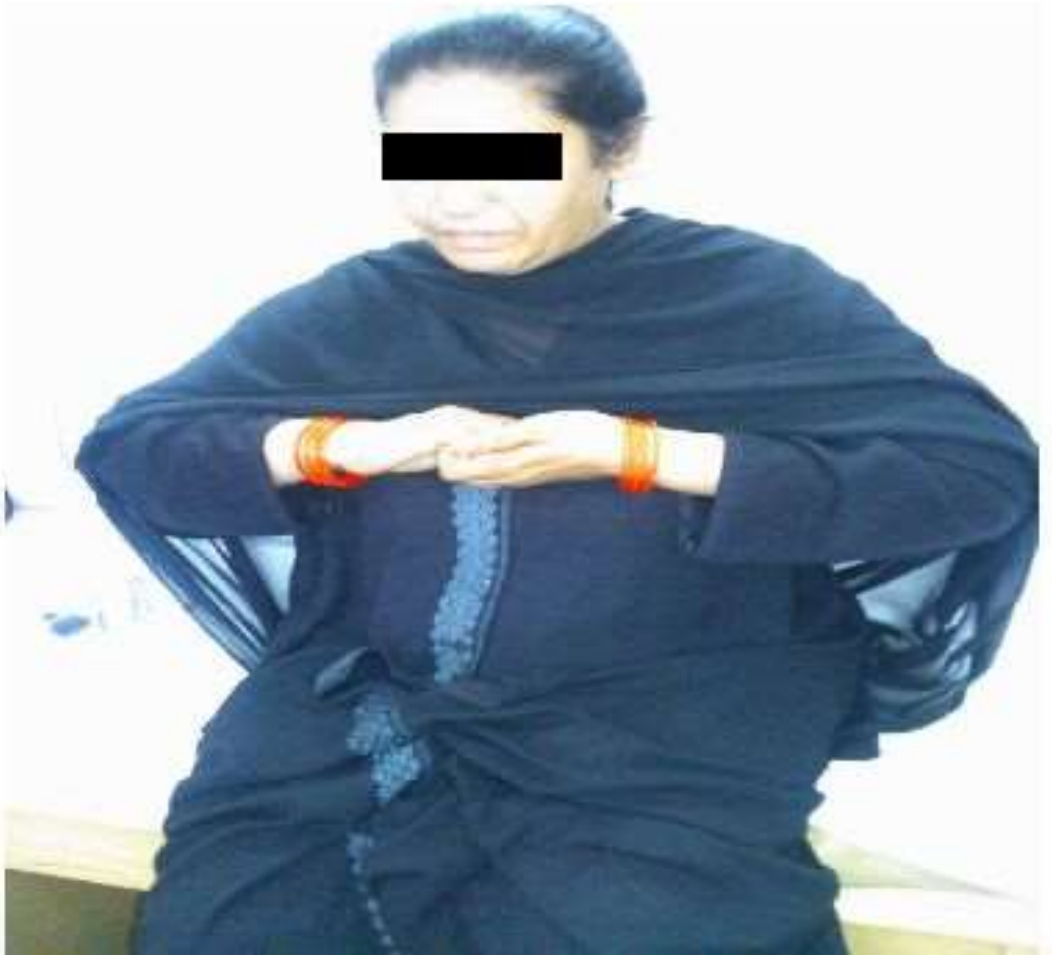


FIG 3.9: ASSESSING DPN OF THE STUDY SUBJECT BY ANKLE REFLEX USING HAMMER

FIG. 3.10 ASSESSING DPN OF THE SUBJECT BY VIBRATION SENSATION USING 128 Hz TUNING FORK

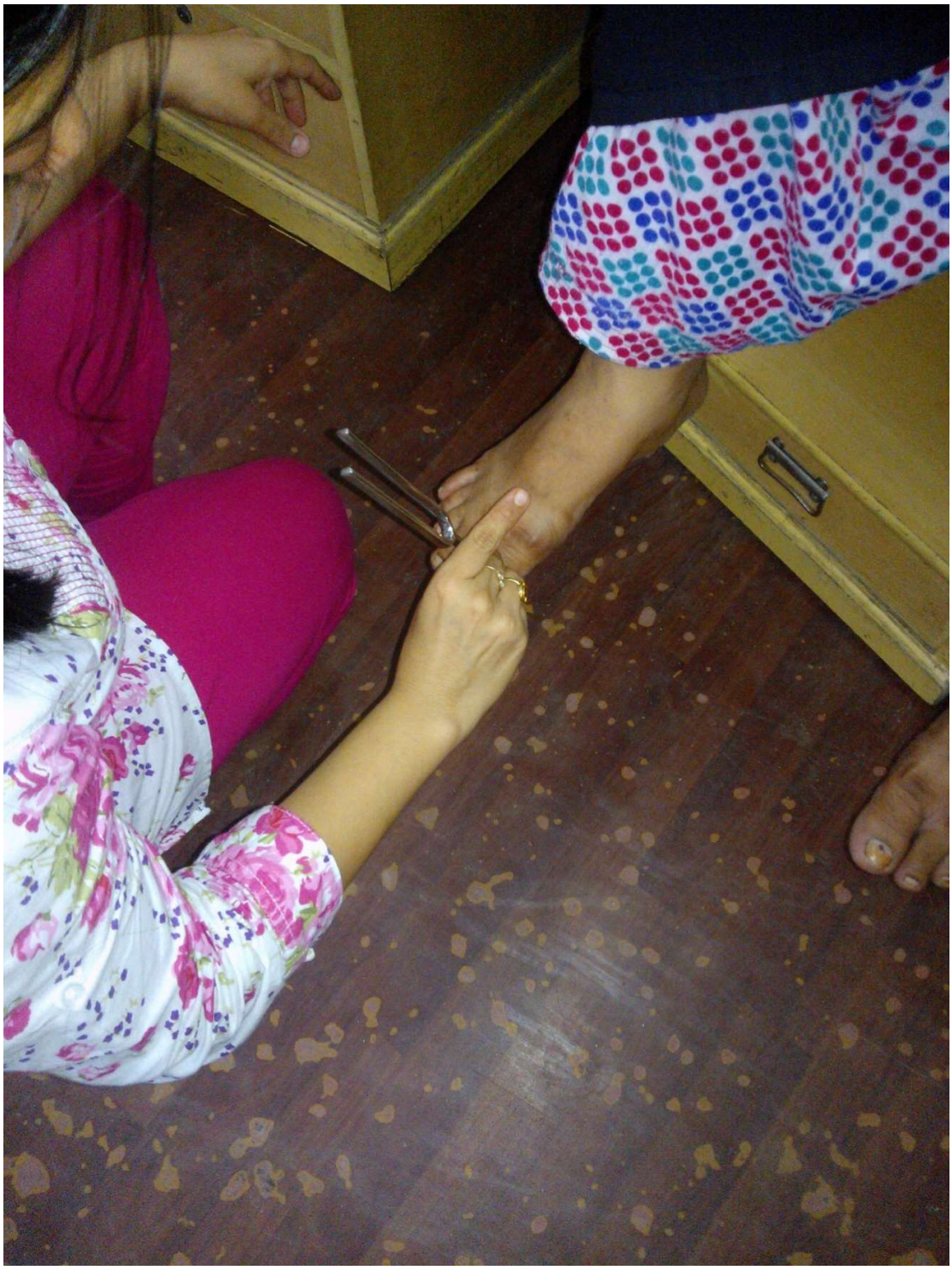


FIG 3.11: ASSESSING DPN OF THE SUBJECT BY MONOFILAMENT TESTING USING 10g MONOFILAMENT

MARKET SURVEY FOR CHOICE OF SUPPLEMENTS AND ADMINISTRATION OF DESIRED DOSE OF B12 AND CALCIUM SUPPLEMENTS (2014):

In the beginning phase of the study, a small market survey was conducted to choose the drugs for vitamin B12 and calcium supplementation in the year 2014.

The market survey was conducted at various pharmacy shops of *Bhagirath* place in Delhi along with the pharmacy shop at Sir Ganga Ram Hospital. While doing market survey for vitamin B12 a number of multivitamin tablets or capsules from reputed brands were reviewed. It was found that majority of the B12 supplements were combination drugs and hence they were not considered (Fig 3.12). Instead the drug with only vitamin B12 component was searched at 'Bhagirath Place' market in old Delhi.

From this survey it was found that sole vitamin B12 were in formulations of methylcobalamine or mecobalamine in various doses of 500µg, 1000µg and 1500µg. To have uninterrupted supplementation a formulation which was economic and whose continuous supply was ensured was desirable. So finally vitamin B12 of 500 µg in name of *Mecobalamine* manufactured by *Rapross Pharmaceuticals Pvt Ltd* (Fig 3.13) was procured. Since the recommended dose was 1000µg per day so the patient was asked to have two tablets in a day, one in morning and the other at night.

As regards calcium supplementation it was found that two formulations of calcium existed in market: calcium carbonate and calcium citrate malate. As reported by majority of the medical representatives the absorption of calcium citrate malate was better than that of calcium carbonate so the choice of calcium citrate malate was made. Sole calcium citrate malate did not exist in market. Rather it was in combination with vitamin D.

Calcium Citrate Malate of 500 mg in name of *Cal 360* manufactured by *Cipla Ltd* (Fig 3.14) was chosen. Since one tablet of Cal 360 contains 250mg calcium citrate+ 400IU D3 and the desired dose of supplementation of calcium was 500mg so two tablets were given to each patient, one in morning and the other at night.

It was ensured that the drugs bought had a good expiry window and its continuous supply was administered. During the course of supplementation, the patients were asked to

preserve the wrappers of consumed tablets. The supplementation was monitored by counting the wrappers of the consumed tablets (Fig 3.15) and there was 100% compliance of supplementation among all the subjects.

MEASURING QUALITY OF LIFE

The Quality of Life was measured among adults with T2DM using WHOQOL BREF questionnaire 2004 (Annexure- VI and Annexure VII).

The WHOQOL-WHOQOL contains a total of 26 questions; the first two questions describing the Overall quality of life and General Health facet have been included. The remaining 24 questions represent each of the 24 facets of which the original instrument is composed (WHOQOL-100), divided into four domains: physical, psychological, social relationships and environment as shown in Table 2.8 in the previous chapter. (WHOQOL-BREF, 1996). The WHOQOL-BREF contains Likert scale with five types of response: “very poor to very good”, “very dissatisfied to very satisfied”, “none to extremely”, “none to complete” and “never to always”. Each domain is made up of questions for which the scores vary between one and five.

The mean score in each domain indicates the individual’s perception of their satisfaction with each aspect of their life, relating it with quality of life. The higher the score, the better this is perceived to be.

A pre-test was conducted with 15 T2DM adults registered in outpatient department of Sir Ganga Ram Hospital using direct interview technique considering problems with vision and low levels of schooling. No difficulty was observed in their understanding of the questionnaire as the questions were also explained in Hindi language for clarity. The translation of the English questions from WHOQOL BREF 2004 to Hindi language was done with the help of Hindi version of WHOQOL BREF 1996 (Annexure VII). The questions in the questionnaire were asked to the patients as to how they feel about your quality of life, health, or other areas of your life. The questions were read out to the patients along with the response options first in English and then in Hindi; if asked by the patient. The patients were asked to choose the answer that appears most appropriate.

FIG 3.12: VITAMIN B12 COMBINATION SUPPLEMENTS AVAILABLE IN THE MARKET SURVEY REJECTED FOR THE STUDY

FIG 3.13: *MECOBAL* - VITAMIN B12 TABLET WITH SOLE B12 FORMULATION SELECTED FOR SUPPLEMENTATION

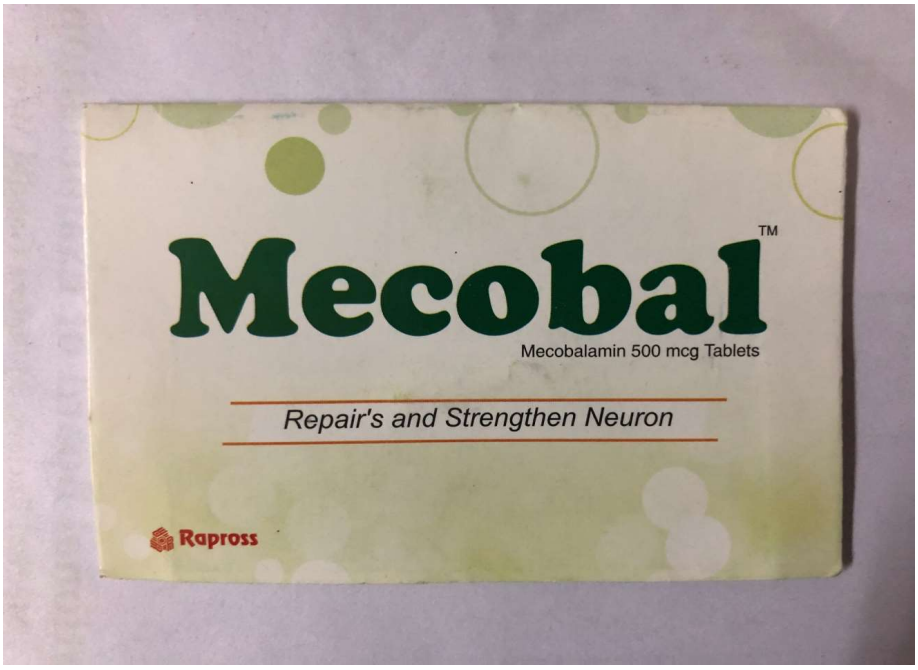


FIG 3.14: *Cal 360*: THE CALCIUM TABLET USED FOR SUPPLEMENTATION

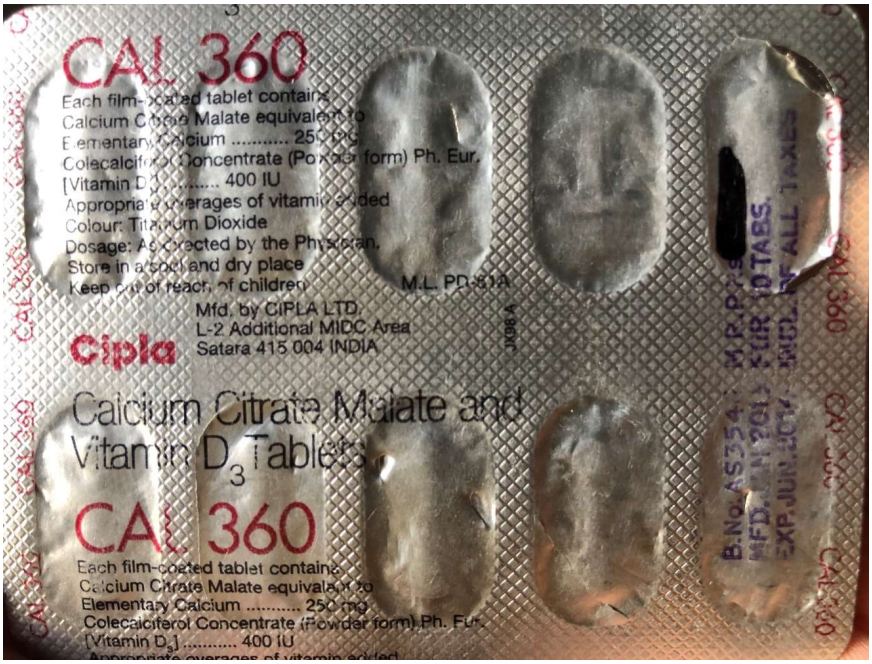


FIG 3.15: SUPPLEMENTATION MONITORED BY COUNTING WRAPPERS OF THE MEDICINE CONSUMED

Patients were explained that if they were unsure about which response to give to a question, the first response which they think of is often the best one. Patients were asked to think about their life in the *last four weeks* and then respond to each question.

STATISTICAL ANALYSIS

The data was entered in Microsoft Excel 2007 and analyzed in SPSS 16.0 version (SPSS, Chicago, IL, USA), Epi Info 7 and Excel 2016. The guide to data analysis using SPSS (Gaur and Gaur, 2006) was used for statistical analysis.

- The quantitative (continuous) variables have been presented as mean±standard deviation.
- Frequencies and percentages have been used to quantify categorical variables.
- Analysis of variance have been performed to obtain F value for continuous variables where more than two groups are compared.
- Pearson correlation have been used to define the strength of the association between variables (+1=perfect positive correlation, -1=perfect negative correlation).
- A confidence interval of 95% have been taken, based on sample observation.
- Bar charts have been used to depict frequency distribution for nominal and ordinal data.
- Chi-square test have been performed to determine association between two categorical variables.
- Students' t-test has been used to compare continuous variables in two groups.
- Box plot was constructed to study distribution of Serum B12 using Excel 2016
- Paired t-test has been used to compare the difference between the pre and post
- data for the outcome variables in the intervention study.
- ODDs ratio have been calculated to estimate the strength of association between two paired dichotomous random variables
- Results have been considered statistically significant with a 2 tailed $p < 0.05$*, $p < 0.01$** and $p < 0.001$***
- Receiver Operating Curve (ROC) analysis have been used to define DPN cut offs.