4. Materials and Methods

This cross sectional study was carried out in the Department of Biochemistry, Sri Ramachandra Medical College and Research Institute, Sri Ramachandra Institute of Higher Education and Research. The study was approved by the Institutional Ethics Committee (IEC-N1/16/JUN/53/36). The written informed consent was obtained from all the study participants.

All study participants answered a self-assessed questionnaire which contains details of age, sex, marital status, detailed history of demography, diet, exercise, smoking, alcohol consumption, menstrual history, medication and history of previous fractures and family history of bone disease were taken.

4.1 Inclusion criteria

For objectives 1 and 2:

Sample size: 170 individuals in the age group of 30-90 years of both sexes, who have undergone DEXA scan for suspected osteoporosis and patients who come with bone fractures to the Orthopaedic Department of Sri Ramachandra Institute of Higher Education and Research, were chosen for the study.

For objective 3:

Sample size: 20 bone marrow biopsy specimens from the osteoporotic fractured patients within the 170 participants.

4.2 Exclusion Criteria

Patients with malignancy, stroke, hemi/paraplegia, chronic kidney and liver disease, rheumatoid arthritis, ankylosing spondylitis, hyperparathyroidism, thyroid disease, chronic smokers and alcoholics, cases of organ transplantation and bed ridden patients, patients on drugs like steroids, Immunosuppressive therapy, antiepileptics, bisphosphonates, hormone replacement therapy, vitamin-D, calcitonin and teriparatide were excluded from the study.
4.3 Study design

![Flowchart of Study Design]

**Figure 4.1 Schematic representation of Study Design**

- **Study Population (n=170)**
  - BMD analysis (DEXA Scan)
  - Group I - Normal Bone Mass (n=57)
  - Group II - Osteopenia (n=62)
  - Group III - Osteoporosis (n=51)

- **Blood Specimens**
  - Whole Blood
  - Isolation of DNA
  - Quantification of DNA
  - PCR-RFLP
    - GENE
      - LRP5
      - AdipoQ
      - COLIA1
  - Agarose Gel Electrophoresis

- **Bone Marrow Biopsy Specimens**
  - Serum
  - Histomorphometry - BMF
  - Basic Biochemical Markers
    - Lipid profile
    - Calcium
    - Phosphorous
    - ALP
    - hsCRP
  - Special Biochemical Markers
    - 25 OH Vitamin D
    - Sclerostin
    - PINP
    - CTX-I
    - NTX-I
    - Adiponectin
    - FGF21
4.4 Anthropometric measurements

- **Height, Weight and BMI**
  The height and weight were recorded in all the study participants and the body mass index (BMI) were calculated by dividing weight (kg) by height (m²).

- **Waist circumference, Hip circumference and waist hip ratio**
  The study participants waist and hip circumference were measured by standard method using stretch resistant measuring tape with 100g tension and waist-to-hip ratio was calculated by waist measurement(inches) divided by hip measurement(inches).

- **Skin fold thickness**
  The skin fold thickness was measured using a skin fold caliper on the abdomen, scapular and triceps region.
  
  Procedure: After explaining the procedure to the study participants, a fold of skin is firmly grasped between the thumb and index finger. The same was lifted up. The lifted skin fold included the skins and the subcutaneous fat, but no muscle or fascia. The contact surface of the caliper was placed at a 90 degree angle to the skin fold approximately 1cm below the fingers. The pressure between the fingers was slightly released, but skin fold was held so that a greater pressure was applied by the caliper. The handle of the caliper was released; the needle to the nearest 0.1 mm was recorded approximately 4 seconds after the pressure was released.

4.5 BMD measurement by DEXA densitometer (GE Lunar Prodigy, Advance Bone Densitometer, US)

  The BMD was determined at the neck of femur (hip) and lumbar spine (L1-L4) by dual energy X-ray absorptiometry (DEXA) densitometer. The DEXA scan was obtained by standard procedure according to manufacturer supplied protocol for scanning and analysis. All the BMD measurements were carried out by the same well-trained technician for all the study participants. Daily quality control (QC) check was carried out by the measurement of Lunar Phantom. This every day QC check provides stable results. The BMD values were expressed as the amount of bone mineral matter per cm² area and the obtained values.
After obtaining the DEXA scan reports. The study subjects were grouped into three, based on the DEXA T score of BMD, according to WHO criteria,

- Group I – Normal bone mass (n=57)
- Group II – Osteopenia (n=62)
- Group III – Osteoporosis (n=51)
4.6 Types of specimen

The 5ml of venous blood was collected without any anticoagulant for biochemical analysis and 2.5ml of EDTA blood was collected for genetic analysis. The bone marrow biopsy specimen was collected during surgery in Groups II and III patients who underwent hip replacement surgeries for fractures.

4.7 Analysis of biochemical markers

➤ The following basic biochemical markers were estimated by AU680., Chemistry system BECKMAN COULTER., analyser

• Lipid profile
  a) Total Cholesterol - Cholesterol oxidase and peroxidase method
  b) TGL - Enzymatic method
  c) HDLc - Polymer-Polyanion method
  d) LDL - LDL direct method

• Calcium - O-Cresolphthaleincomplexone method
• Phosphorous - Modified Daly and Ertingshausen’s method
• ALP - pNPP-AMP method
• Hs CRP - Particle enhanced turbidimetric immunoassay method

➤ The special biochemical markers were estimated by

• 25 OH Vitamin D - Chemiluminescence microparticle enhanced immunoassay
• Sclerostin - ELISA
• PINP - ELISA
• CTX-I - ELISA
• NTX-I - ELISA
• Adiponectin - ELISA
• FGF21 - ELISA
4.7.1 Estimation of total cholesterol by cholesterol oxidase and peroxidase method

**Principle**

Cholesterol esterase hydrolyses cholesterol esters into free cholesterol and fatty acids. In the second reaction cholesterol oxidase converts cholesterol to cholest-4-en-3-one and hydrogen peroxide. In the presence of peroxidase, hydrogen peroxide oxidatively couples with 4-aminoantipyrine and phenol to produce red quinoneimine dye which has absorbance maximum at 540-600nm. The intensity of the red colour is proportional to the amount of total cholesterol in the sample.

![Chemical Reaction Diagram](image)

**Reagents**

R1 reagents: Buffer (pH 6.8), cholesterol oxidase, cholesterol esterase, peroxidase, 4-amino antipyrine, phenol.

**Procedure**

Aspirate 3μL of sample and 350μL of R1 reagent were added. The method is End point method.

**Calculation**

The intensity of the colour is proportional to total cholesterol concentration. Absorbance at 540-600 nm is determined and a standard curve between the concentrations of cholesterol against the absorbance is obtained.

- **Reference range**: < 200mg/dL
- **Linearity**: 25-700 mg/dL
- **Sensitivity**: 25 mg/dL
4.7.2 Estimation of triglyceride by enzymatic method

**Principle**

Glycerol released from hydrolysis of triglycerides by lipoprotein lipase is converted by glycerol kinase into glycerol 3 phosphate which is oxidized by glycerol phosphate oxidase to dihydroxyacetone phosphate and hydrogen peroxide. In presence of peroxidase, hydrogen peroxide oxidizes phenolic chromogen to a red coloured compound.

```
Lipoprotein lipase
Triglycerides    ➔   Fatty acids + Glycerol

Glycerol  kinase
Glycerol + ATP ➔ Glycerol 3 phosphate + ADP

Glycerol phosphate oxidase
Glycerol 3 phosphate + O₂ ➔ Dihydroxyacetone phosphate + H₂O₂

Peroxidase
H₂O₂ + phenolic chromogen ➔ Red colour compound
```

**Reagents**

R1 reagent: Buffer (pH 7.2), Lipase, Glycerol kinase, Glycerol phosphate oxidase, Peroxidase, ATP, Chromogen.

**Procedure**

Aspirate 3μL of serum sample and 350μL of R1 reagent were added. The method is endpoint method.

**Calculation**

The intensity of the colour is proportional to triglyceride concentration. Absorbance at 520 nm was determined and a standard curve between the concentrations of triglyceride against the absorbance is obtained.

**Reference range**: < 150 mg/dL

**Linearity**: 10-1000 mg/dL

**Sensitivity limit**: 10 mg/dL
4.7.3 Estimation of high density lipoprotein Cholesterol (HDLc) by Polymer-Polyanion method

Principle

In this method a magnesium/dextran sulfate solution is first added to the specimen to form water-soluble complexes with non-HDL cholesterol fractions. These complexes are not reactive with the measuring reagents added in the second step. With addition of reagent 2, HDL-cholesterol esters are converted to HDL-cholesterol by PEG-cholesterol esterase. The HDL-cholesterol is acted upon by PEG-cholesterol oxidase, and the hydrogen peroxide produced from this reaction combines with 4-aminoantipyrine and HSDA under the action of peroxidase to form a purple/blue pigment that is measured photometrically at 600 nm (secondary wavelength = 700 nm). When the cholesterol measuring enzymes are modified with PEG, they are preferentially more reactive with HDL-cholesterol than the other cholesterol fractions. This is an endpoint reaction that is specific for HDL-cholesterol.

Reagents

- R1 reagents: HEPES buffer, CHES pH 7.4, dextran sulfate, magnesium nitrate hexahydrate, HSDA, asorbate oxidase, peroxidase and preservative. No preparation required.
- R2 reagents: HEPES buffer, pH 7.0, PEG-cholesterol esterase, PEG-cholesterol oxidase, peroxidase, 4-amino-antipyrine, preservative. No preparation required.

Procedure

Aspirate 5µL of serum sample and 300µL of R1 reagent were added then 100µL of R2 reagent were added. The method is endpoint method.

Calculation

The intensity of the colour absorbance at 600 nm is determined and a standard curve between the concentrations of HDL-Cholesterol against the absorbance is obtained.

| Reference range | : >40 mg/dL |
| Linearity      | : 2.5 – 200 mg/dL |
| Sensitivity limit | : 2.5 mg/dL |
4.7.4 Estimation of low density lipoprotein cholesterol (LDLc) by direct enzymatic method

Principle

The Direct determination of serum low-density lipoprotein cholesterol (LDLc) levels without the need for any pre-treatment or centrifugation steps. The assay takes place in two steps.

1. Elimination of lipoprotein without –LDL:

\[
\text{Cholesterol esterase} \quad \text{Cholesterol esters} \rightarrow \text{Cholesterol + Fatty acids}
\]

\[
\text{Cholesterol oxidase} \quad \text{Cholesterol + O}_2 \rightarrow 4 \text{cholestenone} + \text{H}_2\text{O}_2
\]

\[
\text{Catalase} \quad 2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2
\]

2. Measurement of LDLc:

\[
\text{Cholesterol esterase} \quad \text{Cholesterol esters} \rightarrow \text{Cholesterol + Fatty acids}
\]

\[
\text{Cholesterol oxidase} \quad \text{Cholesterol + O}_2 \rightarrow 4 \text{cholestenone} + \text{H}_2\text{O}_2
\]

\[
\text{Peroxidase} \quad 2\text{H}_2\text{O}_2 + 4\text{-aminoantipyrine} + 3\text{-methylaniline} \rightarrow 4\text{H}_2\text{O} + \text{Quinoneimine}
\]

The intensity of the color formed is proportional to the LDLc concentration in the sample.

Reagents

- R1 reagent: Buffer (pH 7.0), cholesterol esterase, cholesterol oxidase, catalase and N-Ethyl-N-3-methylaniline.
Materials and Methods

- R2 reagent: Buffer (pH 7.0), 4-aminoantipyrine and peroxidase

Procedure

Aspirate 4μL of serum sample and 300μL of R1 reagent were added. Mix and incubate for 5 minutes at 37°C. To this 100μL of R2 reagent is added and incubate for 5 minutes at 37°C. The method is endpoint and the reaction is positive reaction, measured at 600nm.

Calculation

The intensity of the colour is proportional to the LDL-Cholesterol concentration. Absorbance at 600 nm is determined and a standard curve between the concentrations of LDL-Cholesterol against the absorbance is obtained.

Results are calculated, usually automatically by the instrument.

Reference range : <100 mg/dL
Linearity : 7.0 – 400 mg/dL
Sensitivity : 7.0 mg/dL
4.7.5 Estimation of serum calcium by O-Cresolphthalein complexone method

Principle

Calcium ions (Ca$^{2+}$) reacting with O-cresolphthalein complexone in an alkaline solution to form an intense violet colored complex which maximally read at 577 nm.

8-Hydroxyquinoline is added to remove interference by magnesium and iron. In this method the absorbance of the Ca-OCPC complex is measured bichromatically at 570/660 nm. The resulting increase in absorbance of the reaction mixture is directly proportional to the calcium concentration in the sample.

\[
\text{Ca}^{2+} + \text{O-CPC} \xrightarrow{\text{8-Hydroxyquinoline}} \text{pH}10.6 \rightarrow \text{Ca-O-CPC Complex (Violet)}
\]

Reagents

- Ethanolamine (pH 10.6)
- O-Cresolphthalein-complexone
- 8-Hydroxyquinoline
- Hydrochloric acid

Procedure

20µL of serum samples were mixed with 500µL of buffer reagent; after incubation for 5 min at 25°C, 500µL of colour reagent is added. Mix and incubate 25°C for 5 minutes. The method is endpoint method and the reaction is positive reaction, measured at 570 nm.

Calculation

The intensity of the colour is proportional to serum calcium concentration. Absorbance at 570 nm is determined and a standard curve between the concentrations of serum calcium against the absorbance is obtained. Results are calculated, usually automatically by the instrument.

Reference range : 8.6 - 10.3 mg/dL
Linearity : 0.13 -18 mg/dL
Sensitivity : 0.13 mg/dL
4.7.6 Estimation of serum phosphorous by Modified Daly and Ertingshausen’s method

Principle

This inorganic phosphorus method is based on a modification of the method developed by Daly and Ertingshausen in which inorganic phosphate reacts with molybdate to form a heteropolyacid complex. The use of a surfactant eliminates the need to prepare a protein free filtrate. The absorbance at 340/380 nm is directly proportional to the inorganic phosphorus level in the sample.

\[
\text{Phosphate} + \text{Molybdate} \xrightarrow{\text{H}^+} \text{Heteropolyacid complex}
\]

Reagents

- Ammoniumheptamolybdate
- Sulphuric acid
- Glycine

Procedure

Aspirate 3μL of serum sample and 150μL of R1 reagent is added. Mix and incubate for 6 minutes. The method is endpoint method and reaction is positive reaction, measured at 340-380 nm.

Calculation

The intensity of the colour is proportional to the serum phosphorus concentration. Absorbance at 340-380 nm is determined and a standard curve between the concentrations of serum phosphorous against the absorbance is obtained.

Reference range : 3.7 – 7.2 mg/dL
Linearity : 1.0 - 20 mg/dL
Sensitivity : 1.0 mg/dL
4.7.7 Estimation of serum alkaline phosphatase (ALP) by pNPP-AMP method

Principle

ALP at an alkaline pH hydrolysis of p-nitrophenylphosphate to form p-nitrophenol and phosphate. The rate of formation of p-nitrophenol is measured as an increase in absorbance which is proportional to the ALP activity in the sample.

\[
\text{ALP} \quad \text{p-nitrophenylphosphate} \rightarrow \text{p-nitrophenol} + \text{phosphate}
\]

Reagents

- Buffer reagents
- Substrate reagents

Procedure

Take 1000μL of working reagent and incubate at the assay temperature for 1 minute. Then add 20μL of serum sample. Mix well and read the initial absorbance (Ao) after 1 minute and repeat the absorbance, reading after every 1, 2 and 3 minutes at 405 nm. Then calculate mean absorbance change per minute.

Calculation

The ALP activity in U/L = mean absorbance per minute \( \times 2754 \)

Results are calculated, usually automatically by the instrument.

Reference range : 34 - 104 U/L

Linearity : 5.0 - 1500 U/L

Sensitivity : 5.0 U/L
4.7.8 Estimation of high-sensitive C reactive protein (hs-CRP) by particle enhanced turbidimetric immunoassay method

Principle

The synthetic particle coated with antibody to C-reactive protein (abPR) aggregate in the presence of C - reactive protein in the sample. The increase in turbidity which accompanies aggregation is proportional to the C-reactive protein concentration.

\[
\text{CRP} + \text{abPR} \rightarrow \text{Aggregate}
\]

(Absorbs at 340 nm)

Reagents

R1 reagent: Anti-CRP coated particle, Glycine, SDS and Microbial inhibitors

R2 reagent: Buffer, PEG and microbial inhibitors

Procedure

Aspirate 2µL of serum sample and 80µl of R1 reagent and 168µL of R2 reagent are added. The method is endpoint method, and the measurement is bichromatic rate measured at 340-700 nm.

Calculation

Results are calculated, usually automatically by the instrument.

Reference Range : <0.3 mg/dL
Measurement range : 0.05 - 80 mg/dL
Analytical sensitivity: 0.05 mg/dL
Interferences: hemolysis sample, lipemia and sample with unconjugated bilirubin above 60mg/dl
4.7.9 Estimation of serum 25-OH vitamin D by chemiluminescent microparticle immunoassay (CMIA) method

Principle

The 25-OH vitamin D assay is a quantitative delayed one-step competitive immunoassay to determine the presence of vitamin D in human serum and plasma using CMIA technology with flexible assay protocols. The sample, assay diluent and paramagnetic anti-vitamin D coated microparticles are combined. 25-OH vitamin D present in the sample is displaced from the vitamin D binding protein and binds to anti-vitamin D coated microparticles, forming an antigen antibody complex. After incubation, a conjugate containing acridinium-labeled vitamin D is added to the reaction mixture and binds to unoccupied binding sites of the anti-vitamin D coated microparticles. After further incubation and washing, pre-trigger and trigger solutions are added to the reaction mixture. The resulting chemiluminescent reaction is measured as relative light units. There is a relationship between the amount of 25-OH vitamin D in the sample and the relative light units detected by the system optics.

Reagents

- Microparticles: Anti-vitamin D IgG (rabbit monoclonal) coated microparticles in MES buffer. Minimum concentration is 0.04% solids
- Conjugate: Acridinium-labeled vitamin D in MES and surfactant. Minimum concentration 12 ng/ml labeled vitamin D
- Assay diluent: Citrate buffer with EDTA, mehonol, 8-amino-1-naphthalenesulfonic acid and surfactant
- Pre-trigger solution: pre-trigger solution containing 1.32% hydrogen peroxide
- Trigger solution: trigger solution containing 0.35 N sodium hydroxide
- Wash buffer: wash buffer containing phosphate buffered saline solution

Assay procedure

- Before loading the reagent kit on the system for the first time, the microparticle bottle requires mixing to resuspendmicroparticle. After the first time the
microparticles have been loaded, no further mixing is required. Invert the microparticle bottle 30 times. Visually inspect the bottle to ensure microparticles are resuspended. If microparticles still adhere to the bottle, continue to invert the bottle until the microparticles have been completely resuspended. Once the microparticles have been resuspended, place a septum on the bottle.

- Load the reagent kit on the ARCHITECT iSystem
  - Verify that all necessary reagents are present.
  - Ensure that septums are present on all reagent bottles
- Order calibration
- Order tests
- Minimum sample cup volume is calculated by the system and printed on the ordered list report. To minimize the effects of evaporation, verify adequate sample cup volume is present prior to running the test. Maximum number of replicates sampled from the same sample cup: 10
- Sample volume: 60μL
- Load samples
- Press RUN

**Calculation**

The ARCHITECT 25 OH vitamin D assay utilizes a 4 parameter logistic curve data reduction method (4PLC, Y-weighted) to generate a calibration curve. With the help of calibration curve the concentration of serum 25 OH vitamin levels were automatically calculated by the instrument.

**Reference Range**: 30-100 ng/mL

**Measurement range**: 3.4 to 155.9 ng/mL

**Analytical sensitivity**: 3.4 ng/mL

**Interference**: lipemia and testing samples from patients, who receiving vitamin D supplementation
Equipment and other components used for the ELISA testing

Equipments required:

- Microplate ELISA reader (Bio-RAD: iMark)
- Microplate ELISA washer (Rayto:RT-2600c)
- Micro pipettes (Eppendorf)

Other requirements:

- Beakers, flasks, cylinders necessary for preparation of reagents
- Distilled water
- Absorbent paper
- Microtips
- Eppendorf tubes
- Loading slot for wash buffer

4.7.10 Quantitative determination of human serum SOST (sclerostin)

The human sclerostin level in serum is estimated using a commercially available enzyme linked immunosorbent assay kit (Elabscience) according to the manufacturer protocol.

Principle

The micro ELISA plate has been pre-coated with an antibody specific to human SOST (sclerostin). Standards or samples are added to the micro ELISA plate wells and combined with specific antibody. Then a biotinylated detection antibody specific for human SOST and Avidin-horseradish peroxidase (HRP) conjugate are added to each micro plate well successively and incubated free components are washed away. The substrate solution is added to each well. Only those wells that were contain human SOST, biotinylated detection antibody and avidin-HRP conjugate will appear blue in color. This enzyme substrate reaction is terminated by the addition of stop solution and the color turns the yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450nm. The OD values are proportional to the concentration of human
SOST. Calculate the concentration of human SOST in the samples by comparing the OD of the samples to the standard curve.

**Requirements**

- Antibody pre-coated micro ELISA plate
- Reference Standard and sample diluent
- Biotinylated detection antibody (100x) and diluent
- HRP conjugate (100x)
- Wash buffer (25x)
- Substrate reagent
- Stop solution

**Reagents preparation**

- Bring all reagents to room temperature before use
- Wash buffer: Dilute 30 mL of concentrated wash buffer into 750 mL of wash buffer with distilled water
- Standard working solution: Centrifuge the standard at 10,000xg for 1 minute. Add 1.0 ml of reference standard diluent, let it stand for 10 minutes and turn it upside down for several times. After it dissolves fully, mix it thoroughly with a pipette. This reconstitution produces a working solution of 4 ng/ml. Then make serial dilutions as needed. The recommended dilution gradient is as follows: 4, 2, 1, 0.5, 0.25, 0.125, 0.062, and 0.00 ng/mL.

Dilution method (standard): Take 7 Eppendorf tubes; add 500 µl of reference standard diluent to each tube. Pipette 500 µl of the 4 ng/ml working solution to the first tube and mix up to produce a 2 ng/ml working solution. Pipette 500 µl of the solution from the former tube to the latter one in order according to this step. The illustration below is for reference.
Materials and Methods

Study of Biomarkers and Gene Polymorphism and their Association with Bone Mineral Density in Patients with Osteoporosis

• Biotinylated detection antibody working solution: calculate the required amount before experiment (100µL/well). Centrifuge the stock tube before use; dilute the 100x concentrated biotinylated detection antibody to 1x working solution by biotinylated detection antibody diluent.

• Concentrated HRP conjugate working solution: calculate the required amount before experiment (100µL/well). Dilute the 100x concentrated HRP conjugate to 1x working solution by HRP conjugate diluent.

Assay procedure

a) 100µL standard working solution of different concentration to the first 7 wells is added and 100µL of serum sample is added to other wells. Incubate all the wells for 90 minutes at 37°C.

b) Remove the liquid of each well and immediately added 100µL of biotinylated detection antibody working solution to each well. Gently mixed up and incubate for 1 hour at 37°C.

c) Aspirate the solution from each well and 350µL of wash buffer is added to each well. Soak for 2 minutes and aspirate the solution from each well and part it dry against clean absorbent paper. Repeat this wash step 3 times in total.

d) 100µL of HRP conjugate working solution is added to each well and incubate for 30 minutes at 37°C.

e) Aspirate the solution from each well and repeat the wash process for five times as conducted in step c.
f) 90µL of substrate reagent is added to each well and incubate for about 15 minutes at 37°C.

g) 50µL of stop solution is added to each well

h) Determine the optical density (OD) of each well at once, using a microplate reader set to 450nm

i) The concentration of serum sclerostin in test sample is determined directly from the standard curve

Calculation of results

Plot a four parameter logistic curve on log to log graph paper, with standard concentration on the X-axis and the OD values on Y-axis. The concentration is calculated from the standard curve. If samples have been diluted, the concentration calculated from the standard curve must be multiplied by the dilution factor.

**Reference range**: Not yet established

**Measurement range**: 0.062 - 4ng/mL

**Analytical sensitivity**: 0.062 ng/mL
4.7.11 Quantitative determination of human serum PINP (Procollagen I N-terminal Propeptide)

Principle

The micro ELISA plate has been pre-coated with an antibody specific to human PINP (Procollagen I N-terminal Propeptide). Standards or samples are added to the micro ELISA plate wells and combined with specific antibody. Then a biotinylated detection antibody specific for human PINP and Avidin-horseradish peroxidase (HRP) conjugate are added to each micro plate well successively and incubated free components are washed away. The substrate solution is added to each well. Only those wells that were contain human PINP, biotinylated detection antibody and avidin-HRP conjugate will appear blue in color. This enzyme substrate reaction is terminated by the addition of stop solution and the color turns the yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450nm. The OD values are proportional to the concentration of human PINP. Calculate the concentration of human PINP in the samples by comparing the OD of the samples to the standard curve.

Requirements:

- Antibody pre-coated micro ELISA plate
- Reference Standard and sample diluent
- Biotinylated detection antibody(100x) and diluent
- HRP conjugate (100x)
- Wash buffer (25x)
- Substrate reagent
- Stop solution

Reagents preparation:

- Bring all reagents to room temperature before use
- Wash buffer: Dilute 30 mL of concentrated wash buffer into 750 mL of wash buffer with distilled water
- Standard working solution: Centrifuge the standard at 10,000xg for 1 minute. Add 1.0 mL of reference standard diluent, let it stand for 10 minutes and turn it upside
and down for several times. After it dissolves fully, mix it thoroughly with a pipette. This reconstitution produces a working solution of 4ng/ml. Then make serial dilutions as needed. The recommended dilution gradient is as follows: 1000, 500, 250, 125, 62.5, 31.25, 15.63, and 0.00 pg/mL.

Dilution method (standard): Take 7 Eppendorf tubes; add 500µL of reference standard diluent to each tube. Pipette 500µl of the 1000 pg/ml working solution to the first tube and mix up to produce a 500 pg/ml working solution. Pipette 500µL of the solution from the former tube to the latter one in order according to this step. The illustration below is for reference.

- Biotinylated detection antibody working solution: calculate the required amount before experiment (100µL/well). Centrifuge the stock tube before use; dilute the 100x concentrated biotinylated detection antibody to 1x working solution by biotinylated detection antibody diluent.
- Concentrated HRP conjugate working solution: calculate the required amount before experiment (100µL/well). Dilute the 100x concentrated HRP conjugate to 1x working solution by HRP conjugate diluent.

Assay procedure

a) 100µl standard working solution of different concentration to the first 7 wells is added and 100µL of serum sample is added to other wells. Incubate all the wells for 90 minutes at 37°C.
b) Remove the liquid of each well and immediately added 100µL of biotinylated detection antibody working solution to each well. Gently mixed up and incubate for 1 hour at 37°C.

c) Aspirate the solution from each well and 350µL of wash buffer is added to each well. Soak for 2 minutes and aspirate the solution from each well and part it dry against clean absorbent paper. Repeat this wash step 3 times in total.

d) 100µL of HRP conjugate working solution is added to each well and incubate for 30 minutes at 37°C.

e) Aspirate the solution from each well and repeated the wash process for five times as conducted in step c.

f) 90µl of substrate reagent is added to each well and incubate for about 15 minutes at 37°C.

g) 50µL of stop solution is added to each well

h) Determine the optical density (OD) of each well at once, using a microplate reader set to 450nm

i) The concentration of serum P1NP in test sample is determined directly from the standard curve.

**Calculation of results**

Plot a four parameter logistic curve on log to log graph paper, with standard concentration on the X-axis and the OD values on Y-axis. The concentration is calculated from the standard curve. If samples have been diluted, the concentration calculated from the standard curve must be multiplied by the dilution factor.

**Reference range**

- Male: 25-70 years: 15-80µg/L, >70 years: 15-115µg/L*
- Female: 25-49 years: 15-70µg/L, >50 years: 15-90µg/L*

*Reference interval for human serum PINP, reproduced from harmonized Australian reference interval by Samuel D vasikaran et al.,

**Measurement range** : 9.38 – 1000pg/mL

**Analytical sensitivity** : 9.38pg/mL
4.7.12 Quantitative determination of human serum NTX-I (Cross Linked N-Telopeptide of Type I Collagen)

The human NTX-I level in serum is estimated using a commercially available enzyme linked immunosorbent assay kit (SiNCERE) according to the manufacturer protocol.

**Principle**

The micro ELISA plate has been pre-coated with purified human NTX-I (Cross Linked N-Telopeptide of Type I Collagen) antibody. Standards or samples are added to the micro ELISA plate wells and combined with human NTX-I antibody which with HRP labeled, become antibody-antigen- enzyme-antibody complex, after washing completely, add TMB substrate solution, TMB substrate becomes blue colour at HRP enzyme-catalyzed, reaction is terminated by addition of a sulphuric acid solution and the colour change (yellow) spectrophotometrically at a wavelength of 450nm. The concentration of human NTX-I in the sample is then determined by comparing the optical density (OD) of the samples to the standard curve.

**Requirements:**

- Antibody pre-coated micro ELISA plate
- Standard 270 ng/ml
- Standard diluent
- Sample diluent
- HRP conjugate
- Wash buffer (30x)
- Chromogen solution A
- Chromogen solution B
- Stop solution

**Assay procedure**

- Preparation of standards: the following is a table that outlines the relationship of strips used and standard mix prepared.
<table>
<thead>
<tr>
<th>Strip no.</th>
<th>Std. Con.,</th>
<th>Human NTX Standards</th>
<th>Human NTX Standard diluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>180ng/mL</td>
<td>100µL</td>
<td>50 µL</td>
</tr>
<tr>
<td>2</td>
<td>120ng/mL</td>
<td>100µL</td>
<td>50 µL</td>
</tr>
<tr>
<td>3</td>
<td>60ng/mL</td>
<td>100 µL</td>
<td>50 µL</td>
</tr>
<tr>
<td>4</td>
<td>30ng/mL</td>
<td>100 µL</td>
<td>50 µL</td>
</tr>
<tr>
<td>5</td>
<td>15ng/mL</td>
<td>100 µL</td>
<td>50 µL</td>
</tr>
</tbody>
</table>

- Set wells separately: Set blank well (did not add sample and HRP conjugate reagent to the blank comparison wells, other each step operation is same), and testing sample well.
- Adding sample: 40µL of sample diluent is added in the testing sample well and 10µL of serum sample is added (Sample final dilution is 5 fold). Then mix gently
- After closing the plate by plate closure membrane. The plate is incubated for 30 minutes at 37°C
- Uncover the closure plate membrane and aspirate the solution from each well and 350µl of wash buffer is added to each well. Soak for 2 minutes and aspirated the solution from each well and part it dry against clean absorbent paper. Repeat this wash step 3 times in total.
- 50µl of HRP conjugate working solution is added to each well, except the blank well
- After closing the plate by plate closure membrane. The plate is incubated for 30 minutes at 37°C
- Aspirate the solution from each well and repeated the wash process for five times as conducted in step 5.
- Then add 50µl of TMB chromogen A and 50µl of TMB chromogen B to each well, mix gently, evade the light and incubate for 15 minutes at 37°C
- 50µL of stop solution is added to each well
• Determine the optical density (OD) of each well at once, using a microplate reader set to 450nm
• The concentration of serum NTX-I in test sample is determined directly from the standard curve.

Calculation of results

Plot a four parameter logistic curve on log to log graph paper, with standard concentration on the X-axis and the OD values on Y-axis. The concentration is calculated from the standard curve. If samples have been diluted, the concentration calculated from the standard curve must be multiplied by the dilution factor.

**Reference range**: Serum NTX-I not yet established

**Measurement range**: 2.54 - 200 ng/mL

**Analytical sensitivity**: 2.54 ng/mL
4.7.13 Quantitative determination of human serum CTX I (Cross linked C-telopeptide of type I collagen)

The human CTX I level in serum is estimated using a commercially available enzyme linked immunosorbent assay kit (Elabscience) according to the manufacturer protocol.

Principle

The micro ELISA plate has been pre-coated with an antibody specific to human CTX-I (Cross linked C-telopeptide of type I collagen). Standards or samples are added to the micro ELISA plate wells and combined with specific antibody. Then a biotinylated detection antibody specific for human CTX-I and Avidin-horseradish peroxidase (HRP) conjugate are added to each micro plate well successively and incubated free components are washed away. The substrate solution is added to each well. Only those wells that were contain human CTX-I, biotinylated detection antibody and avidin-HRP conjugate will appear blue in color. This enzyme substrate reaction is terminated by the addition of stop solution and the color turns the yellow. The optical density (OD) is measured spectrophotometrically at a wavelength of 450nm. The OD values are proportional to the concentration of human CTX-I. Calculate the concentration of human CTX-I in the samples by comparing the OD of the samples to the standard curve.

Reagents used for the testing:

- Antibody pre-coated micro ELISA plate
- Reference Standard and sample diluent
- Biotinylated detection antibody(100x) and diluent
- HRP conjugate (100x)
- Wash buffer (25x)
- Substrate reagent
- Stop solution

Reagents preparation

- Bring all reagents to room temperature before use
Materials and Methods

Study of Biomarkers and Gene Polymorphism and their Association with Bone Mineral Density in Patients with Osteoporosis

- Wash buffer: Dilute 30 mL of concentrated wash buffer into 750 mL of wash buffer with distilled water
- Standard working solution: Centrifuge the standard at 10,000xg for 1 minute. Add 1.0 mL of reference standard diluent, let it stand for 10 minutes and turn it upside and down for several times. After it dissolves fully, mix it thoroughly with a pipette. This reconstitution produces a working solution of 4 ng/ml. Then make serial dilutions as needed. The recommended dilution gradient is as follows: 10, 5, 2.5, 1.25, 0.63, 0.32, 0.16, and 0.00 ng/mL.

Dilution method: Take 7 Eppendorf tubes; add 500 µL of reference standard diluent to each tube. Pipette 500 µL of the 10 ng/ml working solution to the first tube and mix up to produce a 5 ng/mL working solution. Pipette 500 µL of the solution from the former tube to the latter one in order according to this step. The illustration below is for reference.

- Biotinylated detection antibody working solution: calculate the required amount before experiment (100 µL/well). Centrifuge the stock tube before use; dilute the 100x concentrated biotinylated detection antibody to 1x working solution by biotinylated detection antibody diluent.
- Concentrated HRP conjugate working solution: calculate the required amount before experiment (100 µL/well). Dilute the 100x concentrated HRP conjugate to 1x working solution by HRP conjugate diluent.
Assay procedure

a) 100µL standard working solution of different concentration to the first 7 wells is added and 100µL of serum sample is added to other wells. Incubate all the wells for 90 minutes at 37°C.
b) Remove the liquid of each well and immediately added 100µL of biotinylated detection antibody working solution to each well. Gently mixed up and incubate for 1 hour at 37°C.
c) Aspirate the solution from each well and 350µL of wash buffer is added to each well. Soak for 2 minutes and aspirate the solution from each well and part it dry against clean absorbent paper. Repeat this wash step 3 times in total.
d) 100µl of HRP conjugate working solution is added to each well and incubate for 30 minutes at 37°C.
e) Aspirate the solution from each well and repeated the wash process for five times as conducted in step c.
f) 90µL of substrate reagent is added to each well and incubate for about 15 minutes at 37°C.
g) 50µL of stop solution is added to each well
h) Determine the optical density (OD) of each well at once, using a microplate reader set to 450nm
i) The concentration of serum CTX 1 in test sample is determined directly from the standard curve.

Calculation of results

Plot a four parameter logistic curve on log to log graph paper, with standard concentration on the X-axis and the OD values on Y-axis. The concentration is calculated from the standard curve. If samples have been diluted, the concentration calculated from the standard curve must be multiplied by the delusion factor.

Reference range  
Male: 25-70 years: 0.1-0.6 ng/mL, >70 years: 0.1-0.75 ng/mL  
Female: 25-49years: 0.15-0.8ng/mL, >50 years: 0.05-0.8ng/mL
Materials and Methods

*Reference interval for human serum PINP, reproduced from harmonized Australian reference interval by Samuel D vasikaran et al.,

Measurement range : 0.16 - 10ng/mL
Analytical sensitivity : 0.1ng/mL

4.7.14 Quantitative determination of human serum total adiponectin

The human total adiponectin level in serum is estimated using a commercially available enzyme linked immunosorbent assay kit (Quantikine, R&D systems Inc.) according to the manufacturer protocol.

Principle

This assay employs the quantitative sandwich immunoassay technique. A monoclonal antibody specific for the human adiponectin globular domain has been pre-coated into a microplate. Standards and samples are pipetted into the wells and any adiponectin present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme linked monoclonal antibody specific for the human adiponectin globular domain is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and colour develops in proportion to the amount of adiponectin bound in the initial step. The colour development is stopped and the intensity of the colour was measured at 450nm.

Reagents used for the testing:

- Monoclonal antibody pre-coated micro ELISA plate
- HRP conjugate
- Human Adiponectin Standard
- Assay Diluent (RD1W)
- Calibrator Diluent
- Wash buffer concentrate (25x)
- Colour Reagent A and B
- Stop Solution
Reagents preparation

Wash Buffer: Add 20mL of wash buffer concentrate to distilled water and to prepare 500ml of wash buffer

Substrate Solution: Colour reagent A and B were mixed together in equal volumes within 15 minutes of use. Protect from light. 200µL of the resultant mixture is required per well.

Human adiponectin standards: Reconstitute the human adiponectin standard with calibrator diluent. This reconstitution produces a stock solution of 250ng/mL. Mix the standard to ensure complete reconstitution and allow the standard to sit for a minimum of 15 minutes within gentle agitation prior to making dilutions.

Pipette 200µL of calibrator diluent into each tube. Use the stock solution to produce a dilution series (bellow). Mix each tube thoroughly before the next transfer. The human adiponectin standard (250ng/mL) serves as a high standard. The appropriate calibrator diluent serves as the zero standard (0.00ng/mL).

Sample preparation

Serum samples require a 100-fold dilution. The suggested 100-fold dilution is 10µL of sample + 990µL of calibrator diluent.

Assay procedure
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**Materials and Methods**

**Study of Biomarkers and Gene Polymorphism and their Association with Bone Mineral Density in Patients with Osteoporosis**

a) Add 100µL of assay diluent to each well

b) Add 50µL of standard, control or samples per well. Incubate for 2 hours at room temperature. A plate layout is provided to record standards and samples assayed

c) Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with wash buffer 350µL using an auto washer. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining wash buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

d) Add 200µL human adiponectin conjugate to each well. Incubate for two hours at room temperature

e) Repeat the aspiration/wash as in step 3

f) Add 200µL of substrate solution to each well. Incubate 30 minutes at room temperature

g) Add 50µL of stop solution to each well. The colour changed from blue to yellow

h) Determine the optical density (OD) of each well within 30 minutes, using a microplate reader set to 450nm. And Calculate the results

**Calculation of results**

Plot a four parameter logistic curve on log to log graph paper, with standard concentration on the X-axis and the OD values on Y-axis. The concentration is calculated from the standard curve. If samples have been diluted, the concentration calculated from the standard curve must be multiplied by the dilution factor.

**Reference range**

Male: 18-49 years: 4.3-19.7µg/mL, ≥50 years: 4.2-28.8 µg/mL*

Female: 18-49 years: 5.7-38.7µg/mL, ≥50 years: 7.0-56.3 µg/mL*

*Reference interval for human serum total adiponectin, reproduced from the study of Joel Estis et al., “comprehensive adult reference interval for circulating adiponectin” by EIA

**Measurement range**: 2.42 - 150 ng/mL

**Analytical sensitivity**: 2.42 ng/mL
4.7.15 Quantitative determination of serum fibroblast growth factor - 21(FGF-21)

The human FGF-21 level in serum is estimated using a commercially available enzyme linked immunosorbent assay kit (Epitope Diagnostics, Inc.) according to the manufacturer protocol.

**Principle**

The assay utilizes the two-site sandwich technique with two selected antibodies that bind to different epitopes of human intact FGF-21. One of the antibodies specifically binds to the N-terminal human FGF-21 and other is specific to the C-terminal FGF-21.

Assay standards, controls and patient samples are added directly to the wells of a microplate that is coated with an anti-human FGF-21 specific antibody. Simultaneously, a horseradish peroxidase-conjugated anti-human FGF-21 specific antibody is added to each well. After the first incubation period, the antibody on the wall of microtiter well captures human FGF-21 in the sample and unbound proteins in each microtiter well are washed away. A sandwich of anti-FGF-21 antibody and human intact FGF-21 HRP conjugated tracer antibody is formed. The unbound tracer antibody is removed in the subsequent washing step. For the detection of this immunocomplex, the well is then incubated with a substrate solution in a timed reaction and then measured in a spectrophotometric microplate reader. The enzymatic activity of the immune complex bound to human intact FGF-21 on the wall of the microtiter well is directionally proportional to the amount of intact FGF-21 in the sample.

**Reagents used for the testing**

- Anti-human FGF-21 antibody coated microplate
- Human FGF-21 Tracer antibody
- FGF-21 Tracer antibody diluent
- Wash buffer concentrate
- HRP substrate
- Human FGF-21 standards
- Human FGF-21 controls
Reagent preparation

- **Wash buffer concentrate**: one bottle contains 30ml of 30-fold concentrate. Before use the content must be diluted with 870ml of distilled water and mixed well. Upon dilution this yield a working wash solution containing a surfactant in phosphate buffered saline with a non-azide, non-mercury preservative.
- **Standards and controls**: reconstitution of standards and controls by adding 0.5ml distilled water into each vial. Gently mix and dissolve the entire particle before use. The reconstituted standards and controls should be stored -20°C right after use.
- **Human FGF-21 tracer antibody**: working human FGF-21 tracer antibody was prepared by 1:21 fold dilution of conjugation antibody with the FGF-21 tracer antibody diluent. Following is a table that outlines the relationship of strips used and antibody mix prepared.

<table>
<thead>
<tr>
<th>Strip no.</th>
<th>FGF-21 tracer antibody diluent</th>
<th>FGF-21 tracer antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>500µL</td>
<td>25 µL</td>
</tr>
<tr>
<td>2</td>
<td>1000 µL</td>
<td>50 µL</td>
</tr>
<tr>
<td>3</td>
<td>1500 µL</td>
<td>75 µL</td>
</tr>
<tr>
<td>4</td>
<td>2000 µL</td>
<td>100 µL</td>
</tr>
<tr>
<td>5</td>
<td>2500 µL</td>
<td>125 µL</td>
</tr>
<tr>
<td>6</td>
<td>3000 µL</td>
<td>150 µL</td>
</tr>
<tr>
<td>7</td>
<td>3500 µL</td>
<td>175 µL</td>
</tr>
<tr>
<td>8</td>
<td>4000 µL</td>
<td>200 µL</td>
</tr>
<tr>
<td>9</td>
<td>4500 µL</td>
<td>225 µL</td>
</tr>
<tr>
<td>10</td>
<td>5000 µL</td>
<td>250 µL</td>
</tr>
<tr>
<td>11</td>
<td>5500 µL</td>
<td>275 µL</td>
</tr>
<tr>
<td>12</td>
<td>6000 µL</td>
<td>300 µL</td>
</tr>
</tbody>
</table>
Materials and Methods

- Test configuration showed in the following table

<table>
<thead>
<tr>
<th>ROW</th>
<th>Strip 1(Conc.)</th>
<th>Strip 2(Conc.)</th>
<th>Strip 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Std-1(0.00pg/mL)</td>
<td>Std-5(752pg/mL)</td>
<td>Sample 1</td>
</tr>
<tr>
<td>B</td>
<td>Std-1(0.00pg/mL)</td>
<td>Std-5(752pg/mL)</td>
<td>Sample 2</td>
</tr>
<tr>
<td>C</td>
<td>Std-2(54pg/mL)</td>
<td>Std-6(2100pg/mL)</td>
<td>Sample 3</td>
</tr>
<tr>
<td>D</td>
<td>Std-2(54pg/mL)</td>
<td>Std-6(2100pg/mL)</td>
<td>Sample 4</td>
</tr>
<tr>
<td>E</td>
<td>Std-3(121pg/mL)</td>
<td>C-1(159pg/mL)</td>
<td>Sample 5</td>
</tr>
<tr>
<td>F</td>
<td>Std-3(121pg/mL)</td>
<td>C-1(159.8pg/mL)</td>
<td>Sample 6</td>
</tr>
<tr>
<td>G</td>
<td>Std-4(291pg/mL)</td>
<td>C-2(404pg/mL)</td>
<td>Sample 7</td>
</tr>
<tr>
<td>H</td>
<td>Std-4(291pg/mL)</td>
<td>C-2(405.7pg/mL)</td>
<td>Sample 8</td>
</tr>
</tbody>
</table>

Assay Procedure

a) Place a sufficient number of antibody coated micro well strips in a holder to run human intact FGF-21 standards, controls and unknown samples in duplicate.

b) Add 50µL of standards, controls and patients serum samples in a designated micro well.

c) Then add 50µL 1:21 diluted tracer antibody to each well.

d) Cover the plate with one plate sealer and incubate plate with orbital shaking 170 rpm at room temperature for 2 hours.

e) Remove the plate sealer and aspirate the content of each well. Washed each well 5 times by dispensing 350µL of working wash solution into each well and then completely aspirate the contents.

f) Add 100µL of HRP substrate into each of the well

g) Cover the plate and also with aluminum foil to avoid exposure to light. Incubate plate at room temperature for 20 minutes.

h) Remove the aluminum foil and plate sealer then add 100µl of stop solution into each of the wells. Mix gently.

i) Read the absorbance at 450/650nm within 10 minutes in a microplate reader.
Calculation of results

A standard curve is generated by plotting the absorbance verses the respective human intact FGF-21 concentration for each standard on point to point or 4 curve fit. The concentration of human intact FGF-21 in test sample is determined directly from this standard curve. If samples have been diluted, the concentration is calculated from the standard curve must be multiplied by the dilution factor.

**Reference range**: Not yet established

**Measurement range**: 1.7–2500 pg/mL

**Analytical sensitivity**: 1.7 pg/mL
4.8 Genetic analysis

The molecular analysis was constituted with the following,

- Isolation of genomic DNA
- Qualitative analysis of isolated DNA
- Quantitative analysis of isolated DNA
- In vitro amplification of gene of interest by polymerase chain reaction
- Identification of single nucleotide gene polymorphism by RFLP
- Agarose gel electrophoresis

4.8.1 Isolation of genomic DNA from peripheral blood leukocytes by phenol–chloroform–isoamyl alcohol (PCI) method

Principle

This is the most commonly used method of purifying and concentrating DNA from blood samples. The DNA is extracted by phenol: chloroform: isoamyl alcohol mixture to remove the protein contaminants and then precipitated with 100% ethanol. The DNA is pelleted after the precipitation step, washed with 70% ethanol to remove salts and small organic molecules and re-suspended in a buffer solution and to get a concentrated isolated DNA, which is suitable for further experimentation.

Testing reagents and preparation

- **1 M Tris-HCl stock solution (1 liter):** Dissolve 121.14g Tris base in 800ml of double distilled water. Adjust the pH to 7.5/8.0 with appropriate volume of concentrated HCl and bring final volume to 1 liter with deionized water. Autoclave and store at room temperature.
- **1 M Potassium Chloride (KCl):** Dissolve 7.45g of KCl in 80ml of double distilled water, after complete dissolution of KCl salt, the final volume was made up to 100ml with double distilled water. Autoclave and store at room temperature.
- **1 M Magnesium chloride (MgCl₂):** Dissolve 40.6g of MgCl₂ in 80ml of double distilled water, after complete dissolution of MgCl₂ salt, the final volume was
made up to 100ml with double distilled water. Autoclave and store at room temperature.

- **0.5 M EDTA disodium salt**: Dissolve 18.6g of EDTA disodium salt in 80ml of double distilled water and 15.20g of NaOH pallets are added and dissolve completely. Adjust the pH 8.0 with 1N HCl and final volume was made up to 100ml with double distilled water. Autoclave and store at room temperature.

- **5 M Sodium Chloride (NaCl)**: Dissolve 29.2g of sodium chloride in 80ml double distilled water and warm to assist dissolution. The final volume is made up to 100ml with double distilled water.

- **TritonX-100**: Commercially available TritonX-100 is used.

- **10% Sodium dodecyl sulphate (SDS)**: 10g of SDS is dissolve in 80 ml of sterile double distilled water gently by slow mixing to avoid frothing. The solution was kept in water bath at 65°C to assist complete dissolution. The final volume is made up to 100 ml and the solution was filtered and sterilized. The solution is stored at 4°C.

- **Tris-Buffer-Saturated Phenol**: Tris-buffer-saturated phenol is used. It is commercially available; store under refrigerated conditions.

- **Phenol; chloroform; isoamyl alcohol mix (25:24:1)**: 25 ml of buffer saturated phenol is mixed with 24 ml of chloroform and 1 ml of isoamyl alcohol to get a final volume of 50 ml (PCI stock solution). The solution is stored in clean air tight dark brown bottle at 4°C.

- **Chloroform; isoamyl alcohol (24:1)**: 48 ml of chloroform is mixed with 2 ml of Isoamyl alcohol. The solution is stored in clean airtight dark brown bottle at 4°C.

- **3 M Sodium acetate**: Dissolve 24.6g of sodium acetate in 80 ml of double distilled water and adjust the pH to 5.2 with glacial acetic acid. The final volume is made up to 100 ml with double distilled water. Autoclave and store at room temperature.

- **Absolute ethanol**: Absolute ethanol is used. It is commercially available and store under refrigerated conditions.

- **70% Ethanol**: 70 ml of absolute ethanol is mixed with 30 ml of sterile double distilled water and stored at 4°C.
➤ **Red blood cell (RBC) lysis buffer**

The buffer is also termed as non- nucleated cell lysis buffer or low salt buffer.

- Tris-HCl 10 mM
- KCl 10 mM
- MgCl₂ 10 mM
- Na₂EDTA 2H₂O 2 mM

All the above chemicals are mixed with sterile double distilled water. The buffer is stored at room temperature.

➤ **White blood cell (WBC) lysis buffer**

The buffer is also known as nucleated cell lysis buffer or high salt buffer.

- Tris-HCl 10 mM
- KCl 10 mM
- MgCl₂ 10 mM
- Na₂EDTA 2H₂O 2 mM
- NaCl 400 mM

All the above chemicals are mixed with sterile double distilled water and store at room Temperature

➤ **Tris-EDTA (TE) buffer (pH 8.0)**

- Tris-HCl 10 mM
- Na₂EDTA 2H₂O 1 mM

All the above chemicals are dissolved with sterile double distilled water

➤ **Tris-acetate-EDTA (TAE) buffer**

- Tris base (0.5 M) 12.1 gm
- Glacial acetic acid 5.71 ml
- Na₂EDTA 2H₂O 18.61 gm
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Make 0.5 M Na$_2$EDTA 2H$_2$O solutions by adding 18.6 gm of the Na$_2$EDTA to 80 ml of sterile water and adjust the pH to 8.0 with NaOH solution mix thoroughly. The final volume is made up to 100ml with double distilled water. Autoclave and store at room temperature.

- **10 ng/ml EtBr**

Dissolve 10 ng of ethidium bromide (EtBr) in 1 ml of distilled water and mixed well for complete dissolution of the dye. The stock solution is stored in aliquots in airtight containers wrapped with aluminum foil.

- **Sample loading dye (6x)**

Take 25 mg of bromophenol blue, 25 mg of xylene cyanol FF and 3 ml of glycerol is added. To this make up to 10 ml with sterile double distilled water. The dye is stored at 4°C.

Procedure

RBC lysis:

- 2–2.5 ml of EDTA blood sample and 5 ml of RBC lysis buffer is added. To this solution 0.1% of Triton X-100 is added and mixes gently.
- The tube containing above mixture is incubated at 37°C water bath for 5 minutes.
- After incubation, it is centrifuged at 2000 rpm for 15 minutes at 4°C.
- The supernatant is discarded and to the pellet, 10 ml of RBC lysis buffer is added and the pellet is suspended well.
- This is centrifuged at 2000 rpm for 15 minutes at 4°C.
- The washing process repeated until a white pelletis obtained.

WBC lysis

- In the pellet, 1 ml of WBC lysis buffer is added. In case, pellet is suspended well in the tube, to mix solution by the help of cyclomixer for lyses of the cells.
- Then add 20 µl of SDS and incubated in a water bath at 55°C for 60 minutes (takes out and shakes mildly for every 15 minutes).
Precipitation and purification of DNA

- After continuation of WBC lysis, the solution is carefully transferred to the 2 ml sterile microfuge tube and mix well with equal volume of phenol: chloroform: isoamyl alcohol mixture (mix vigorously using cyclomixer).
- This solution is centrifuge at 10000 rpm for 15 minutes at 4°C.
- After centrifugation the upper aqueous phase is carefully transferred into another tube and equal volume of chloroform: isoamyl alcohol mixture is added, then mix well and spun at 10000 rpm for 15 minutes at 4°C (mix vigorously using cyclomixer).
- The upper phase is carefully transferred into another tube and to this 10 µl of 3M sodium acetate (pH 5.2) is added, mixes gently and double the volume of ice cold absolute ethanol is added. These content are mixed gently by inverting the tube. After some time the DNA precipitate are obtained.
- This is centrifuged at 5000 rpm for 5 minutes to settle down the precipitated DNA at the bottom of the tube and the supernatant is discarded, the DNA pellet is washed with 500 µl of 70% ethanol and centrifuge at 2000 rpm for 15 minutes at 4°C.
- After centrifugation the supernatant is discarded and the DNA pellet is kept for air-dry after this air dried DNA is dissolved in 200 µl of TE buffer. The DNA is aliquoted and store in frozen condition for further experiment.

4.8.2 Qualitative analysis of isolated DNA

The quality of the isolated DNA is checked in 1% agarose gel electrophoresis.

- 1 gram of agarose and 100 ml of 1X TAE buffer is dissolved in a conical flask and this solution is boiled to form a gel.
- Add 0.1 µg/ml concentration of ethidium bromide (gel loading dye) to agarose gel in warm state and gently mix it.
- The gel is poured on a gel-casting tray with comb and allowed to solidify.
- The solidified gel is immersed in an electrophoretic chamber with 1X TAE buffer.
Materials and Methods

The DNA samples are mixed with bromophenol blue (sample loading dye). The 20μl of dye mixed DNA sample is applied in the well created by comb in the gel.

The current is applied at 2 volts/cm in an electrophoretic chamber and the gel is run.

View the gel under UV light. High molecular weight DNA is too large to migrate well under these conditions, whereas degraded DNA contains a spectrum of smaller fragment sizes that appear as a smear across the lane.

4.8.3 Quantitative analysis of isolated DNA

The spectrophotometric analysis was used for the assessment of purity of the isolated DNA. In this study the quality and quantity of the isolated DNA is checked by Nano-drop spectrophotometer.

- The 2 μl of isolated DNA samples are diluted with 998 μl of TE buffer.
- Place the diluted DNA sample in a quartz microcuvet and measure the absorbance at 260 and 280 nm against water blank. (Nucleic acids absorb light maximally at 260 nm whereas proteins absorb strongly at 280 nm). The sample is read at 260 nm and 280 nm.
- Compute the DNA concentration based on the concept that an OD at 260 nm of 1 OD corresponds to 50 μg/mL of double-stranded DNA.
- The OD at 260nm :OD at 280 nm ratio should be between 1.7 and 2.0. Lower values indicate protein contamination, in which case the DNA can be further purified by additional phenol/chloroform extractions followed by ethanol precipitation.
- The total isolated DNA samples are 170, but the good quantity and quality of DNA obtained only 150 samples. Therefore we precede genetic analysis only 150 isolated DNA samples.
4.8.4 Determination of LRP5 genotype

The LRP5 gene that is also known as Ala1330Val; the more common (C) allele encodes the Ala (alanine), while the rarer (T) allele encodes the Val (valine), which is the risk allele. LRP5 gene SNP (Genbankrs3736228) is located in the chromosome 11. The LRP5 gene is genotyped by PCR-RFLP; the forward and reverse primers are used to generate a region of 143 bp in the LRP5 gene that could be used in the subsequent genotyping reactions of the SNP (rs7574865) polymorphism. The DraIII restriction enzyme and band size were described by Zhang ZL et al.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Primers</th>
<th>Band size (bp)</th>
<th>Restriction enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>LRP5</td>
<td>F: 5’-GACTGTCAGGACCCTACAGC-3’</td>
<td>143 bp</td>
<td>DraIII</td>
</tr>
<tr>
<td>(rs3736228)</td>
<td>R: 5’-AAGGTGTTTCAGAGCCCTAC-3’</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The DNA amplifications of the related regions are carried out in 20 μl volumes of each reaction mixtures containing 10 μl master mix buffer (TaqDNA polymerase, buffer, and dNTPs), 3 μl template DNA, 0.3 μl forward primer, 0.3 μl reverse primer and 6.4 μl sterile water. The DNA amplifications are performed in gradient thermocycler (eppendorf, Master cycler, Germany) according to the following protocol:

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>94°C</td>
<td>5 minutes</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>1 minute.</td>
</tr>
<tr>
<td>Annealing</td>
<td>56°C</td>
<td>1 minute.</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C</td>
<td>1 minute.</td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>7 minutes</td>
</tr>
<tr>
<td>Hold at 4°C</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The newly amplified 143 bp DNA fragment is digested with 2 U of allele-specific DraIII restriction endonucleases at 37°C for 8 hours.
The *DraIII* digested products were run on 3% agarose gel electrophoresis with 1x TAE buffer along with 100 bp ladder. The restriction endonuclease *DraIII* cleaves the DNA strand which contains the CT variant gives two bands of 143 bp and 119 bpsizes, however the presence of CC and TT allele is not digested by *DraIII* and gives a single band. The results of electrophoresis are viewed under ultraviolet illuminator and photographed using a UVP Gel Documentation System using VisionWorks® software.

### 4.8.5 Determination of AdipoQ genotype

The AdipoQ gene, SNP (Genbankrs266729) is located in the chromosome 3. The AdipoQ gene are genotyped by PCR-RFLP, the forward and reverse primers are used to generate a region of 334 bp in the AdipoQ gene that could be used in the subsequent genotyping reactions of the SNP (rs266729) polymorphism. The *HhaI* restriction enzyme and band size are described by F. Khabour et al.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Primers</th>
<th>Band size (bp)</th>
<th>Restriction enzyme</th>
</tr>
</thead>
</table>
| *AdipoQ* (rs266729) | F: 5’-GGTGGACTTGACTTTACTGG-3’  
R: 5’-TAGAAGCAGCCTGGAGAA-3’ | 334            | *HhaI*            |

The DNA amplifications of the related regions are carried out in 20 μl volumes of each reaction mixtures containing 10μl master mix buffer (TaqDNA polymerase, buffer, and dNTPs), 3μl template DNA, 0.3μl forward primer, 0.3μl reverse primer and 6.4μl sterile water. The DNA amplifications are performed in gradient thermocycler (eppendorf, Master cycler, Germany) according to the following protocol:

<table>
<thead>
<tr>
<th>Steps</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>94°C</td>
<td>4 minutes</td>
</tr>
</tbody>
</table>
| Denaturation  
Annealing  
Extension   | 94°C  
58°C  
72°C | 45 Sec.  
1 minute.  
1 minute. |
| Final extension | 72°C        | 5 minutes |
| Hold at 4°C   |             |         |
The newly amplified 334 bp DNA fragment is digested with 2 U of allele-specific \textit{HhaI} restriction endonucleases at 37°C for 8 hours.

The \textit{HhaI} digested products are run on 3\% agarose gel electrophoresis with 1x TAE buffer along with 100 bp ladder. The restriction endonuclease \textit{HhaI} cleaves the DNA strand which contains the CG variant gives two bands of 212 bp and 122bpsizes, however the presence of CC and GG allele is not digested by \textit{HhaI} and gives a single band. The results of electrophoresis are viewed under ultraviolet illuminator and photographed using a UVP Gel Documentation System using VisionWorks® software.

4.8.6 Determination of COLIA1 genotype

The COLIA1 gene, SNP (Genbankrs1800012) represents a common polymorphism consisting of a G to T substitution at the first base of a consensus site for the transcription factor Sp1 in the first intron of the COLIA1 gene. The gene is located in the chromosome 17. The COLIA1 gene is genotyped by PCR-RFLP; the forward and reverse primers are used to generate a region of 260 bp in the COLIA1 gene that could be used in the subsequent genotyping reactions of the SNP (rs1800012) polymorphism. The \textit{MScI} restriction enzyme and band size are described by M. Erduran et al.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Primers</th>
<th>Band size (bp)</th>
<th>Restriction enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>COLIA1</td>
<td>\textit{F: 5'}-TAACCTCTGGACTATTTGGGACTT-3'}</td>
<td>260</td>
<td>\textit{MScI}</td>
</tr>
<tr>
<td>(rs1800012)</td>
<td>\textit{R: 5'}-GTCCAGCCCTCATCCTGGCC-3'}</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The DNA amplifications of the related regions are carried out in 20 μl volumes of each reaction mixtures containing 10μl mastermix buffer (TaqDNA polymerase, buffer, and dNTPs), 3μl template DNA, 0.3μl forward primer, 0.3μl rewers primer and 6.4μl sterile water. The DNA amplifications are performed in gradient thermocycler (Eppendorf, Master cycler, Germany) according to the following protocol:
Materials and Methods

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>94°C</td>
<td>3 minutes</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C</td>
<td>50 Sec.</td>
</tr>
<tr>
<td>annealing</td>
<td>62°C</td>
<td>10 Sec.</td>
</tr>
<tr>
<td>extension</td>
<td>72°C</td>
<td>15 Sec.</td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C</td>
<td>5 minutes</td>
</tr>
<tr>
<td></td>
<td>Hold at 4°C</td>
<td></td>
</tr>
</tbody>
</table>

The newly amplified 260 bp DNA fragment is digested with 2 U of allele-specific MScI restriction endonucleases at 37°C for 8 hours.

The MScI digested products are run on 3% agarose gel electrophoresis with 1x TAE buffer along with 100 bp ladder. The restriction endonuclease MScI cleaves the DNA strand which contains the GT variant gives two bands of 260 bp and 242 bp sizes, however the presence of GG and TT allele is not digested by MScI and gives a single band. The results of electrophoresis are viewed under ultraviolet illuminator and photographed using a UVP Gel Documentation System using VisionWorks® software.
4.9 Bone marrow fat analysis

The bone marrow fat quantification process includes the following steps,

- Tissue fixation
- Decalcification
- Tissue processing
- Staining
- Microscopy

4.9.1 Tissue fixation

Principle

- To prevent changes such as putrefaction and autolysis by inhibiting autolytic enzymes and killing the organism that cause decomposition
- To preserve the various tissue constituents as nearly as possible to their original form
- To harden the naturally soft tissue, permitting easier and safer manipulation during subsequent processing.
- To render various tissue constituents receptive to subsequent staining

Types of sample

Excision bone biopsy specimen, which are kept in fixative immediately after removal

Equipment and Reagent required

- Measuring cylinder
- Funnel
- Formaldehyde
- Distilled water
- Phloxin
- Preparation of 10% formalin: prepare the 5 liters of 10% formalin, take 500ml of formaldehyde (37%) and dilute it with 4.5 liters of water. Mix carefully: avoid spill and exposure
Procedure

The fresh tissue is immersed in the fixing solution 10 times the approximate volume of the tissue as soon as possible. For small biopsy specimen the tissue fully left in fixative without any manipulation. For large biopsies, specimen are sliced and immersed in fixative 10 times the volume of specimen. Cotton may be inserted in between the slices for better penetration of fixative. The time for which specimen is kept in fixative depends on the size of the specimen. The 10% formalin is used as a fixative, which penetrates at the rate of 1mm/2hours at room temperature.

4.9.2 Decalcification

Purpose

Removing the calcium salts from the bony tissue and making them amenable for sectioning

Principle

Decalcification involves the use of diluted acids in which the calcium salts dissolves

Type of sample

Well fixed bone tissue of adequate size and thickness

Equipment and reagents required

- Bone saw
- Chisel
- Hammer
- Formic acid *
- Hydrochloric acid*
- Nitric acid*

Reagent preparation

*10% aqueous formic acid: add formic acid (90%) 100ml and distilled water up to 900ml.
*10% Hydrochloric acid: add 90ml distilled water and 10ml concentrated hydrochloric acid
*Nitric acid – formaldehyde: the 10 ml of nitric acid is added 5-10 ml of formalin with distilled water up to 100ml.
Procedure

- Suspend the formalin fixed tissue slice place in a cassette in the decalcifying solution. The cassette is suspended in large quantity of decalcifying solution (more than 20 times the volume of the tissue) for process of decalcification.
- Change the fluid daily, Stirring or agitation of the fluid hastens decalcification.
- The tissue is mechanically or chemically tested for adequate decalcification
- These include probing of tissue block by needle
- Surface decalcification: when a block has been trimmed to reveal the tissue surface, small foci of calcium may occasionally be removed. If this encountered the block can be removed from the microtome and place the cut surface down on a pad of cotton wool saturate with 10% HCl for approximately one hour.

4.9.3 Tissue processing

Purpose

To make tissue ready to be cut 5µm thick (4 µm thick wherever applicable)

Principle

The aim of tissue process is to embed the tissue in a solid medium firm enough to support the tissue and give it optimum rigidity, thus causing no damage to knife to tissue during microtomy.

Types of sample

Well-fixed tissue, for bone specimen, after complete decalcification

Equipment and reagents required

- Instrument: Leica-ASP300
- 10% formalin saline*
- Alcohol
- Xylene
- Molten wax (melting point 58- 60°C
- *10% formalin saline preparation: 0.85 grams of sodium chloride dissolved in 100ml of distilled water.
Procedure

Basic information about the processing:

Dehydration: paraffin is not miscible with water; hence removal of water from the tissue section is essential before paraffin embedding. This must be carried out by the use of reagent, which is mixed with water thus penetrating easily between the tissue cells. Total dehydration time depends on the volume and types of the tissue and dehydration agents. The amount of agent used in each stage should not be less than 10 times the volume of the tissue to be dehydrated.

Dehydration procedure

This process include, passing the tissue through a serious of progressively more concentrated alcohol baths. It is done by the automatic tissue processor. Concentration of first alcohol bath depends on the fixation and size and type of tissue. Routinely 70% alcohol is employed as the first solution.

Clearing

After dehydration, tissue is lightly bloated and transferred into a clearing agent if hand processing is done. Volume of clearing agent should be 10-20 times the volume of specimen. After the appropriate time interval, transfer the tissue from one regent to another. At least two changes should be given in reagent like xylene, for a particular duration and then transferred to impregnating bath.

Impregnation

The paraffin wax is routinely used as the impregnating and embedding media. Paraffin wax remains popular due to ease with which large number of tissue blocks can be processed.

In this study we used paraffin blocks (Emerck/Paxmy) of 58 – 60°C melting point were used in tissue impregnation.
Automated procedure

Programmed: Overnight processing

Tissue fixation – 4 hours (10% formalin saline)

Dehydration:

- 70% ethanol – 60 minutes
- 80% alcohol – 60 minutes
- 90% alcohol – 60 minutes
- 100% alcohol I – 60 minutes
- 100% alcohol II – 60 minutes
- 100% alcohol III – 60 minutes

Clearing:

- Xylene I – 45 minutes
- Xylene II – 45 minutes
- Xylene III – 60 minutes

Wax Impregnation:

- Molten wax I – 1 hour
- Molten wax II – 1 hour
- Molten wax III – 1 hour 30 minutes

4.9.4 Preparation of tissue for staining

- Embedding allows specimen orientation and secures the specimen in a block of wax for section cutting and storage
- Sectioning is done on a microtome that cuts very fine section (2 – 7 µm thick) which is floated out on the water bath then picked up and placed on microscope slide.
- The tissue on the slide is now ready for staining

Staining

- The first staining step is dewaxing which uses solvent to remove the wax from the slide prior to staining. This is always done as part of the staining process. When a
stain is complete the section is covered with a cover glass that makes the preparation permanent.

- The hematoxylin and eosin staining is used routinely

4.9.5 Histological analysis of adipose tissue and quantification of fat in marrow

- After the completion of staining procedure, the stained specimen is placed on the microscope. The average adipocyte area, number and average diameter of fat tissue is identified for each specimen and this is enumerated under high power on 10 constructive microscopic fields.

- The quantity of fat in marrow tissue was calculated by following formula,

\[ = \left( \frac{4\pi}{3} \right) \times R_1 \times R_2 \times R_3 \]

4.10 Statistical analysis

The statistical analysis were performed using SPSS version 20.0 (IBM SPSS V20, USA) for windows. The continuous variables were expressed as mean ± SD. Comparison of normally distributed independent variables were performed using one way ANOVA with Turkey’s HSD post hoc test for identification of differences between specific groups. Receiver operating characteristics (ROC) curve analysis was performed for determination of cut-off point by sensitivity and 1-specificity. Pearson correlation coefficient was used to examine association of BMD, biomarkers, gene polymorphism and bone marrow fat. The levels of p<0.05 were considered statistically significant.