CHAPTER V

METHODOLOGY
5.1 Study Setting:

The present study was held in the Department of Cardiology, Kasturba Medical College Hospital, Manipal University, Manipal, Karnataka, India.

5.2. Duration of the Study:

The data collection began in December 2013 until November 2016; the data analysis was done between December 2016 to February 2017.

5.3. Ethics:

The present study was initiated after the approval of the Institutional Ethics Committee (Approval no IEC 681/2013) (Annexure attached). The patient’s information sheet was provided to all patients and explained the details and risks involved in the study (described in their local language) (Annexure attached). Written informed consent was obtained from all the study participants before study commences (Annexure attached).

5.4. Study populations:

- Group A: Amlodipine induced pedal edema (AIPE), (n=70)
- Group B: Amlodipine treated non- edema (ATNE), (n=70)
- Group C: Cilnidipine treated group (CTG) (Group C is the one-month follow-up group A), (n=70)

5.5. Study Design: The present study contains two phases

- Phase I: Observational Study
  - Comparison of group A and group B
- Phase II: Interventional study
  - Comparison of group A and group C
5.6. **Eligibility criteria for the study:**

### 5.6.1. Inclusion criteria for group A:

Patients included for the study with following reasons:

- Hypertensive patients of either gender (DBP>90 & SBP>140 mm Hg)
- Patients currently receiving amlodipine more than six months for the treatment of HTN
- Age limit, 18 to 70 years
- Patient who develop amlodipine induced pedal edema (with no other apparent cause)

### 5.6.2. Inclusion criteria for group B:

Patients included for the study with following reasons:

- Hypertensive patients of either gender (DBP>90 & SBP>140 mm Hg)
- Patients currently receiving amlodipine more than six months for the treatment of HTN
- Age limit, 18 to 70 years
- Patient who did not develop amlodipine induced pedal edema

**Note:** The group C is the one-month follow-up of group A. Therefore eligibility criteria remained same as group-A.

### 5.6.3. Exclusion criteria for group A and B:

Patients excluded for the following reasons:

- The patients were with major organ failure like kidney, liver and cardiac diseases.
- Endocrine abnormalities Cushing’s syndrome, hypothyroidism
- All gravid women and women on oral contraceptive pills.
Methodology

- Patients receiving α or β blockers, Non-steroidal anti-inflammatory drugs, Steroids
- Lymphedema, Pulmonary Hypertension, Secondary hypertension
- Patients on sympathetic agonist or antagonist
- Patients on any other group of antihypertensive drugs
- Patients with varicose vein, venous insufficiency, obesity and sleep apnea

5.7. Sample size calculations:

The primary outcome variable considered for sample size calculation was Fresno test of EBP competency.

The formula was used was “sample size for comparison for comparison of two means.”

\[
 n = \frac{2 \left( Z_{1-\alpha} + Z_{1-\beta} \right)^2 S^2}{d^2}
\]

- \( Z_{1-\alpha} = 1.96 \) at 5% level of significance.
- \( Z_{1-\beta} = 1.28 \) for 90% power.
- \( S^2 \) = Pooled variance calculated from the pilot study. (3.5 x 3.5 = 12.25)
- \( d^2 \) = clinically significant difference. (2 x 2 = 4)

The minimum required a sample size of 65 (n) in each of the groups i.e. Amlodipine induced pedal edema (AIPE) and amlodipine treated non-edema (ATNE) group. In the present study 70 patients were enrolled in each arm.

5.8. Phase-I study:

Outline of Phase I observational study:

Amlodipine induced pedal edema patient group or group A, (n=70)  Observational study  Amlodipine treated non-edema patient group or group B, (n=70)
5.8.1. Phase-I study procedure:

The phase-I study was a prospective, observational study. The hypertensive patients of either gender attending the outpatient department of cardiology and medicine was recruited in this study. The consultant cardiologist examined the patients, and BP was measured using standard mercury sphygmomanometer. Three consecutive readings of BP and PR were noted at an interval of 10 min. Pedal edema was confirmed by history and examining both the legs. After initial screening, a detailed history was recorded from both the study groups regarding duration of amlodipine induced pedal edema, diabetes, the familial status of diabetes, treatment of type 2 diabetes mellitus, smoking and alcohol status, dyslipidemia, hypertension and familial history of coronary artery disease. Clinical examination findings and biochemical parameters were also noted. All the patients were receiving a dosage of amlodipine 5mg/day; all recruited patients completed the study. A total 140 mild to moderate hypertensive patients (HTN classified according to JNC-7 HTN guideline), 70 were in AIPE group (group-A), and other 70 patients were in ATNE group (group- B).

5.8.2. Statistical Analyses (Phase-1):

Continuous variables were stated as the mean ± Standard deviation (SD) or median with interquartile range (IQR). Categorical variables were reported as the frequency with a percentage. Univariate logistic regression analysis was performed to determine the association of clinic-demographic and laboratory variables with amlodipine induced pedal edema. Further, Variables with p <0.2 in univariate logistic regression analysis were included in the multivariate regressing model with Forward Wald method. Receiver operating curve (ROC) analysis was performed including variables showing significant association with AIPE in univariate logistic regression analysis. Variables having an area under the curve > 0.7 in ROC were identified as well
predicted for AIPE, and a cut off highest sensitivity and specificity was reported. All tests of significance were two-tailed, with p < 0.05 showing statically significant. All the data’s were analyzed by using Statistical Package for Social Sciences (SPSS software version 15), (South Asia, and Bangalore, India).

5.9. Phase-II study:

Outline of Phase II interventional study, (n=70)

- **Amlodipine induced pedal edema (AIPE), or Group A**
  - Baseline demographic, clinical and biochemical parameters were noted
  - Stop amlodipine therapy and substituted equipotent dose of cilnidipine
  - Followed up for one month
  - Reassessed the baseline parameters

5.9.1. Phase-II study procedure:

The phase II study was a prospective, interventional follow-up study. Baseline clinical and biochemical parameters were noted in all 70 patients who belong to group A (phase I), and after initial screening, amlodipine 5mg/day was changed to an equipotent dose of cilnidipine (10mg/day). The consultant cardiologist did the intervention. Followed up for one month and reassessed all the parameters, and all patients have completed the study.
5.9.2. **Statistical Analyses (Phase-II):**

Continuous variables analyzed using Paired t-test or Wilcoxon test and were expressed as the mean ± Standard deviation (SD) or median with interquartile range (IQR). Categorical variables were expressed as the frequency with a percentage. p <0.05 was considered as statistically significant. Data analysis were carried out using Statistical Package for Social Sciences (SPSS software version 15).

5.10. **Demographic parameters:**

A detailed history was taken from all study participants regarding age, gender, patient diseases history and duration, education status, other diseases and its medications, alcoholic status, menstrual status in women, lifestyle, and familial history of coronary artery disease.

5.11. **Anthropometric parameters (284):**

5.11.1. **Height:**

Measured against a vertical board with an attached metric rule, and a horizontal headboard was brought in contact with an uppermost point on the head. It was recorded barefoot, with the person standing on the flat surface positioned so that the line of vision is straight to the body. The arms were hanging freely by the sides, and the head back and heels were in contact with the vertical board. The individual was asked to breathe in deeply and maintained a fully erect position. The top-most point on the head with sufficient pressure to compress the hairs was taken as height.

5.11.2. **Weight:**

Weight was recorded without footwear with light clothes worn on the body, standing straight in the center of weighing machine with body evenly distributed between the foot by the ISI certified weighing machine to the nearest of 100gms.

5.11.3. **Body mass index (BMI):**
BMI was calculated as Weight (kg)/Height$^2$ (Mt)

**Normal Values:** 18-23 kg/m$^2$ (For Asian Indian population)

5.11.4. **Ankle Circumference:**

The subject was seated on a chair; their knees were in full extension and ankle at the neutral position. Ankle circumference was measured by using simple marking tape and measured from the middle point between the articular projection of the anterior tibial tendon and the lateral malleolus (285) (Fig.11). The measurement was repeated three times in each subject, and the mean value was noted. The ankle circumference measured in both the ankles.

![Ankle Measurement](image1.png)

*Figure 11. Measurement of amlodipine induced pedal edema*

5.12. **Clinical parameters:**

5.12.1. **Blood Pressure (BP):**

BP was measured by using well-calibrated and maintained mercury sphygmomanometer. Before BP measurement, each patient was advised to rest for 5 minutes in the supine or sitting position. The arm was supported and positioned at heart level, and any tight cloth was removed. Appropriate cuff size was selected. The cuff was inflated over the brachial artery until disappearance of the pulse, followed by deflation of the cuff until the pulse re-appeared and this was recorded as estimated SBP.
The cuff was re-inflated 30 mm Hg above the predicted SBP, and a stethoscope was placed. After that, the cuff was deflated at the rate of 2 mm Hg per second until the appearance of a rhythmic sound (SBP). Deflation was continued until disappearance of the sound (DBP). The blood pressure was measured three times, and the mean value was recorded.

**Normal value:** 140/90 mmHg.

**5.12.2. Pulse rate:**

Radial artery pulse rate was counted for 1 min.

**Normal value:** 60-80 bpm.

**5.12.3. Electrocardiogram (ECG):**

The electrocardiogram is the graphical representation of the electrical activity of the heart. The heart muscle has a property of automatic rhythmic contraction. The impulse that arises in the conducting system spreads throughout the myocardium resulting in excitation of the heart muscle fibers this results in weak electric currents which spread through the entire body, and it can be recorded by placing electrodes at various positions on the body and connecting them to an electrocardiographic apparatus. An ECG recording was a simple procedure. Where, 12 soft electrodes with a gel placed on the subject’s chest region, arms, and legs. These electrodes were attached to electrical leads and then to the ECG machine. During the test, subjects were advised to lie on a table, while the device records the electrical activity of the heart and places the information on a graph. The patient was advised to keep calm breath regularly and not to talk during the test. From ECG graph QT interval was noted and QTc values re calculated by Bazett’s formula. ECG was recorded by experienced ECG technicians of Kasturba Hospital.
5.12.4. **Echocardiography (ECHO):**

The study participants underwent an echocardiographic examination to assess cardiac structure and functional status and were performed using Vivid 7 Echo machine (GE healthcare system). Left ventricular function assessment was done by using area length method for ejection fraction calculation. Doppler ECHO was incorporated to evaluate cardiac hemodynamics and valvular forward flow. Inferior vena cava (IVC) dimension was measured using m-mode echocardiography to appreciate the phasic variation with the sample placed 10mm adjacent to IVC- Right atrial junction. The maximum and minimum diameter were measured during expiration and inspiration respectively. IVC diameter assessment and its collapsibility can determine central venous pressure and is also an indicator of venous return to the heart. ECHO test was done by experienced sonographers of Kasturba Hospital

5.13. **Biochemical parameters:**

5.13.1. **Blood sample collection:**

Blood samples were collected by venipuncture technique. Total six milliliters of blood sample were collected in BD Vacutainer (3ml in red capped Vacutainer and 3ml in Lavender capped Vacutainer). Before collection of blood samples, the patient was advised to keep calm and relax on the bed for 30 min in the supine position. The blood samples of lavender capped vacutainer (EDTA) used for estimation of plasma renin, vasopressin, and an atrial natriuretic peptide by ELISA kit method. The collected blood samples centrifuged immediately for 10 minutes at 5000 rpm at 4-8°C temperature. Immediately after centrifugation process plasma was separated and aliquoted and stored a deep freezer at -70°C for further analysis. The collected samples were processed in ELISA kit method as instructed by the manufacturer. Repeated freeze-thaw cycles were avoided. For other routine biochemical test, the 3 ml blood was
collected in red capped vacationer (without anticoagulant), later it was allowed to clot for 30 min. The blood was then centrifuged at 3500 rpm for 10 minutes. The top yellow layer of serum was separated out and used for estimation. Blood samples were processed in Cobas 501 autoanalyzer in a clinical biochemistry laboratory, Kasturba Hospital, Manipal. The serum samples were stored at -20°C for further use.

5.13.2. Urine sample collection:

The entire 24-hour Urine sample was collected in a special brown colored glass bottle, which containing 10 ml of 11 N HCl. It was advised to keep in the refrigerator or a cool place during the collection period. The bottle was labeled with subject’s name, collected date, urine volume and time of completion. This sample was utilized for estimation of twenty-four-hour VMA, proteinuria and urinary electrolytes. The urine samples were stored at -20°C for further use.

5.13.3. Vanillyl mandelic acid (VMA):

5.13.3.1. Principle:

The urine extracted with ethyl acetate from the highly acidic sample, purified by extraction into acetate buffer and diazo color reaction will be developed.

5.13.3.2. Procedure:

Glass stoppered tubes of 50 ml volume (4 tubes) were taken and marked as Blank (B), Standard (STD), Control (C) and Test (T). Pipette up 3ml of urine sample and control urine into Test (T) and Control (C) stoppered tube respectively. Added 0.5 ml of 6 N HCL and 1.1g of NaCl in both test and control stoppered tubes, mixed well. 18 ml of ethyl acetate added to both test and control mixed well and discarded the lower layer. 5ml of water was added to each test tubes and control shake well and discard the water layer. Added 9 ml of acetate buffer shake for five min. and discarded the upper tier. 6 ml of buffer extract was added to test and control stoppered tubes. Acetate buffer
was added 6 ml in Blank (B), and 5.25 ml in Standard stoppered tubes. Working standard was 0.75 ml, 1 ml di-azo reagent and 1 ml potassium bicarbonate was added in all the stoppered tubes and mixed well. 6 ml chloroform added in all stoppered tubes shook for five min and discarded the upper layer. 6 ml of 0.2M NaOH added in Blank (B), Standard (STD), Control (C), and Test (T). Stoppered tubes and shake for five minutes. The upper layer transferred into a test tube and measure absorbance at 510 nm.

5.13.4 Proteinuria Test:

5.13.4.1 Test principle:

Proteinuria is used to evaluate diseases involving the kidney, liver, cardiac and thyroid disease all of which are important causes of edema.

5.13.4.2 Turbid metric method:

The collected blood sample was preincubated in an alkaline solution containing EDTA, which denatures the proteins and eliminates interference from magnesium ions. Benzethonium chloride was added to produce turbidity. In this method plasma total protein, albumin, globulin also estimated.

5.13.5 Plasma Osmolality:

Plasma osmolality measures solute particles present in the fluid medium. Osmolality and osmolarity are technically different, but functionally they are same. If the solvent is pure water, there is almost no difference between osmolality and osmolarity. Osmolarity is the number of moles per liter of solution and Osmolality is the number of moles per kg of solvents. Osmolality measured by an analytical instrument called as osmometers. It works on the principle of depression of freezing point.

5.13.6 Ion-Selective Electrode (I.S.E):
The ISE method used for measurements of sodium in the blood and urine samples. When plasma used, lithium heparin, ammonium heparin, and lithium oxalate are suitable anticoagulants. Hemolysis does not cause any change in the values. Twenty-four-hour urine sample is used to measure urinary sodium. ISE is the most commonly used method in clinical laboratories.

5.13.7. Jaffe's Method:
This colorimetric method is used to determine creatinine levels in blood and urine.

5.13.7.1. Principle:
The addition of creatinine and picric acid forms creatinine picrate. The reaction is carried out up to fixed time points to minimize interference of other substances reacting with picric acid. Two ml of creatinine added to 2 ml of saturated picric acid solution and few drops of 10%NaOH perform control with water in case of creatinine solution. Note the orange color with creatinine and yellow color in the other.

5.14. Enzyme-linked Immunosorbent Assay (ELISA):

5.14.1. General Principle:
Enzyme-linked immunosorbent assay is a non-isotopic immune assay. It is based on immunochemical principles of the antigen-antibody reaction. The antibody against the protein to be measured is fixed on the inert solid surface. The proteins to be estimated from the biological sample were poured on the antibody coated surface. The protein-antibody complex is then reacted with a second protein specific antibody, to which an enzyme is covalently linked. These enzymes must be easily assessable and produce preferably colored products. Peroxidase amylase and alkaline phosphatase commonly used. After removing the unbound antibody linked enzyme, the enzyme bound to the second antibody is assayed. The enzyme activity is determined by the action of the enzyme on a substrate to form a product and can be related to the
concentration of protein. The Colour change in each well is measured photometrical using an appropriate wavelength on a plate reader (Multiscan EX, Thermo Electron Corporation, UK). The plate reader is programmed to draw a standard curve and infer the concentration of the plasma samples from this curve using each relative optical density (OD).

5.14.2. **Estimation of plasma renin:**

The human renin ELISA kit is an in vitro enzyme-linked immunosorbent assay for the quantitative measurement of human renin in serum. In this assay employs an antibody specific for human renin coated on a 96-well plate. When the plasma samples pipetted into the wells, Renin that is present in a sample bound to the wells by the immobilized antibody. The wells were washed, and biotinylated anti-human renin antibody added. After washing away unbound biotinylated antibody, HRP conjugated streptavidin pipetted into the wells. Wells are again washed. A TMB substrate solution added to the wells and color changes in proportion to the amount of renin-bound. Stop Solution changes its color from blue to yellow. The intensity of the color measured at 450 nm.

5.14.3. **Reagents:**

- 96 wells Renin Micro plate (12 strips x 8 wells) coated with anti-Human Renin
- Concentrated wash buffer solution (20X)
- Vials of human renin (standard protein)
- Vials of biotinylated anti-Human Renin (Detection Antibody Renin)
- Concentrated HRP-conjugated streptavidin
- Tetramethylbenzidine (TMB) substrate in buffer solution.
- Stop Solution 0.2 M sulfuric acid
- Concentrated assay diluent buffer
5.14.4. **Reagent Preparation:**

All reagents and samples temperature were brought to room temperature (18 - 25°C) before use. Assay diluent was diluted 5-fold with deionized or distilled water before use.

5.14.5. **Sample dilution:** 1X Assay Diluent was used for dilution of plasma samples.

5.14.6. **Preparation of standard:**

Briefly spin a standard protein vial and reconstitute, add 400µl 1X Assay Diluent (assay diluent should be diluted five-fold with deionized or distilled water before use) into standard protein containing vial to prepare a 100 ng/ml standard solution. Dissolve the powder thoroughly by a gentle mix. Pipette 270 µl standard assay diluent into each tube. Use the 100 ng/ml standard solution to produce a dilution series. Shook each tube thoroughly before making next transfer. Standard Assay Diluent serves as the zero standards (0 ng/ml). Standards are prepared just before the use. Storage of Reconstituted standards avoided.

![Figure 12. Plasma renin standard preparations](image)

5.14.7. **Preparation of wash buffer:**

The wash concentrate (20X) contained visible crystals, warm to room temperature and mixed smoothly until dissolved. Dilute 20 ml of wash buffer
Concentrate into deionized or distilled water to yield 400 ml of 1X wash buffer. After using a partial plate diluted wash buffer stored at 2-8° C.

5.14.8. **Detection antibody solution preparation:**

Briefly, spins the detection antibody vial before use. Add 100 µl of 1X assay diluent to the ampoule to prepare a detection antibody concentrate and gently mix the content (the concentrate can be stored at 4°C for five days). Detection antibody concentrate was diluted to 80-fold with the 1X assay.

5.14.9. **HRP-Streptavidin solution preparation:**

The required amount of HRP-Streptavidin solution was prepared freshly just before use. Briefly, spin the HRP-Streptavidin concentrate vial and mix them gently before use, as precipitates can be formed during storage. HRP-Streptavidin concentrate was diluted 500-fold with 1X assay diluent. (Add 20 µl of HRP-Streptavidin concentrate into a tube with 10 ml 1x assay diluent).

5.14.10. **Assay procedure:**

1. All the reagents and test samples were brought to room temperature (18 - 25°C) before use.

2. 100 µl of each standard or sample pipetted into appropriate micro plate wells, covered properly and incubated at room temperature about 2.5 hours with proper shaking.

3. Solution present in the wells discarded and washed for four times with washing buffer to 300 µl each well by using a multi-channel pipette. In each step, the solution is entirely removed to obtain an accurate result. After the last wash, wash buffer removed by aspirating or decanting. Invert the micro plate and blotted against the clean paper towel to ensure the complete dryness of wells.
4. 100 μl of biotinylated antibody was added to each well and incubated for 1 hour at room temperature with gentle shaking.

5. 100 μl of freshly prepared streptavidin solution is pipetted into each microplate wells and incubated for 45 minutes at room temperature with proper shaking.

6. 100 μl of TMB one-step substrate reagent added to each micro plate well and incubated at room temperature for about 30 minutes in the dark with gentle shaking.

7. In the end, 50 μl of stop solution added to each well and reading was taken at 450 nm in ELISA micro plate reader immediately.

5.14.11. Typical standard graph:

The standard curve was constructed by plotting the absorbance OD (Y-Axis) of standards against the known concentration (X- axis) of standards ranging from 0.1, 1.0, 10, 100, 1000 ng/ml in logarithmic scale, using the four parameter algorithm. Here the microplate reader performed the automatic calculations on analyte concentration. The result read as the total concentration of plasma renin in ng/ml.

![Figure 13. Standard graph of plasma Renin](image)
5.15. Estimation of the Atrial natriuretic peptide (ANP):

The ANP Enzyme Immunoassay (EIA) Kit is an in an in-vitro quantitative assay for the detection of ANP peptide based on the competitive enzyme immunoassay principle. Here, a biotinylated ANP peptide was spiked into samples and standards. Later they were added to the plate, were biotinylated ANP competing with endogenous (unlabeled) ANP for the binding to an anti-ANP antibody. After washing, any bound biotinylated ANP interacts with horseradish peroxidase (HRP)-streptavidin, catalyzes a color change reaction. The intensity of the colorimetric signal is directly proportional to the amount of captured biotinylated ANP and inversely proportional to the amount of endogenous ANP in the standard or samples. A standard curve of known concentration of ANP peptide can be established, and the concentration of ANP peptide in the samples can be calculated accordingly. The estimation of ANP in plasma carried out by ELISA Kit method. The standard range of ANP in blood is 25–60 pg/ml.

5.15.1. Reagents:

- 96 wells ANP Microplate (12 strips x 8 wells) coated with secondary antibody(Item A)
- Concentrated wash buffer solution (20X)(Item B)
- Vials of lyophilized ANP peptide (standard protein)(Item C)
- Vials of lyophilized anti-ANP polyclonal antibody (anti-ANP) (Item N)
- Concentrated Assay diluent (item E)
- Vials of lyophilized biotinylated ANP peptide (Item F)
- Concentrated HRP-conjugated streptavidin (Item G)
- Bottles of lyophilized positive control (Item M)
- Tetramethylbenzidine (TMB) substrate in buffer solution (Item H)
- Stop Solution 0.2 M sulfuric acid (Item I)
- Concentrated assay diluent buffer

5.15.2. **Reagents preparations:**

All the ELISA kit reagents were kept on ice during reagent preparation steps.

5.15.3. **Preparation of Plate and Anti-ANP Antibody:**

1. Before opening the sealed micro plate pouch equilibrate to room temperature
2. Label removable 8-well strips as appropriate for our experiment.
3. 5X Assay Diluent B (Item E) was diluted 5-fold with deionized or distilled water.
4. Briefly centrifuged the anti-ANP antibody vial (Item N) and reconstituted with 55 μl of 1X Assay Diluent B to prepare the antibody concentrate and mixed properly.
5. Anti-ANP antibody working solution prepared by diluting the antibody concentrate to 100-fold with 1X Assay Diluent.

5.15.4. **Preparation of Biotinylated ANP:**

The biotinylated ANP vial was briefly centrifuged and reconstituted with 20 μl of double distilled water.

5.15.4.1. **The preparation of working stock solution:**

Transferring entire contents from biotinylated ANP ampoule into a tube which contains 10 ml of 1X assay diluent B and mixed thoroughly. The final concentration of Biotinylated ANP was 20 pg/ml.

5.15.4.2. **Second dilution Biotinylated ANP for standards:**

2ml of working stock solution added to 2 ml of standard assay diluent. The final concentration of Biotinylated ANP was ten pg/ml.

5.15.4.3. **Second dilution Biotinylated ANP for positive control:**
100 μl of working stock solution was added to 100 μl of the prepared Positive Control. The final concentration of Biotinylated ANP was ten pg/ml.

**5.15.4.4 Second dilution Biotinylated ANP for test samples:**

125 μl of working stock solution was added to 125 μl of prepared sample. The final concentration of Biotinylated ANP was ten pg/ml.

**5.15.5. Preparation of Standards:**

1. Labelled 6 micro tubes with the subsequent concentrations like; 1000 pg/ml, 100 pg/ml, 10 pg/ml, 1 pg/ml, 0.1 pg/ml and 0 pg/ml. 450 μl of biotinylated ANP working solution added into each tube, except the 1,000 pg/ml.

2. Briefly, centrifuge the vial of ANP Standard. Reconstitute with ten μl of double distilled water and briefly vortexed. Eight μl of ANP standard and 792 μl of 10 pg/ml biotinylated ANP working solution added into the tube labeled 1000 pg/ml. Mixed thoroughly. This solution works as the first standard (1000 pg/ml ANP standard, ten pg/ml biotinylated ANP).
3. 100 pg/ml standard prepared by pipetting 50 μl of the 1000 pg/ml ANP standard into the tube labeled 100 pg/ml. Mixed thoroughly.

4. Repeat this step with each successive concentration; the dilution series showed in the illustration below. Each time, used 450 μl of biotinylated ANP and 50 μl of the prior concentration until the 0.1 pg/ml reaches. Mix every tube thoroughly before the subsequent transfer.

![Dilution Series Illustration](image)

Figure 15. Plasma ANP standard preparations

5.15.6. Positive Control Preparation:
Briefly centrifuged the positive control vial and reconstituted with 105 μl of double distilled water and 105 μl of working stock solution was added. The final concentration of Biotinylated ANP was ten pg/ml.

5.15.7. Sample preparation:
125 μl of test sample added to 125 μl working stock solution (Biotinylated ANP). Total volume was 250 μl. It was enough for duplicate wells on the micro plate. The final concentration of biotinylated ANP was ten pg/ml.

5.15.8 Preparation of wash buffer:
The wash concentrate (20X) contains visible crystals, warmed to room temperature and mixed thoroughly until it dissolved. The 20 ml of wash buffer concentrate diluted into yield 400 ml of 1X wash buffer by using deionized or distilled water. After using a partial plate diluted wash buffer stored at 2-8° C.

5.15.9. HRP-Streptavidin solution preparation:

The required amount of HRP-Streptavidin solution was prepared freshly just before use. HRP-Streptavidin concentrated vial spin and mixed gently before the use, as precipitates may form during storage. HRP-Streptavidin concentrate should be diluted to 50-fold with 1X assay diluent.

5.15.10. Assay procedure:

1. All the reagents kept on ice during the reagent preparation steps.

2. 100 μl of Anti-ANP antibody added to each well. Incubated for 1.5 hrs at room temperature with gentle shaking (1-2 cycles).

3. Discarded the solution and washed for four times with washing buffer to 300 μl each well by using a multi-channel pipette. In each step, the solution is entirely removed to obtain an accurate result. After the last wash, wash buffer was completely removed by aspirating or decanting. Invert the micro plate and blotted against the clean paper towel to ensure the complete dryness of wells.

4. 100 μl of each standard, positive control, and the sample was added to appropriate micro plate wells, including blank. Covered wells and incubated for 2.5 hrs at room temperature with gentle shaking (1-2 cycles).

5. Discarded the solution and washed for four times as explained above.

6. 100 μl of freshly prepared streptavidin solution is pipetted into each micro plate wells and incubated for exact 45 minutes at room temperature with gentle shaking.
7. Discarded the solution and washing procedure followed as mentioned above.

8. 100 μl of TMB one-step substrate reagent added to each microplate well and incubated at room temperature in the dark for 30 minutes gentle shaking (1-2 cycles).

9. In the end, 50 μl of stop solution added to each well and reading was taken at 450 nm in ELISA micro plate reader immediately.

**5.15.11. Typical standard graph:**

The standard curve was constructed by plotting the absorbance OD (Y-Axis) of standards against the known concentration (X-axis) of standards ranging from 0.01, 0.1, 1, 10, 100, 1000, 10000 pg/ml in logarithmic scale, using the four parameter algorithm. Here the micro plate reader performed the automatic calculations on analyte concentration. The result read as the total concentration of plasma renin in pg/ml.

![Figure 16. Standard graph of ANP](image-url)
5.16. Estimation of vasopressin (VAS):

The vasopressin (VAS) enzyme immunoassay (EIA) kit is an in-vitro quantitative assay based on the competitive enzyme immunoassay principle for detecting Vasopressin peptide. In this test, a biotinylated vasopressin peptide is spiked into the samples and standards. The samples and standards are then added to the plate, where the biotinylated vasopressin peptide competes with endogenous vasopressin for binding to the anti-vasopressin antibody. After a wash step, any bound biotinylated Vasopressin then interacts with horseradish peroxidase (HRP)-Streptavidin, which catalyzes a color change reaction. The intensity of the colorimetric signal which is directly proportional to the amount of captured biotinylated vasopressin peptide and also inversely proportional to the amount of endogenous vasopressin in the standard or samples. By establishing a standard curve of known concentration of vasopressin peptide, the concentration of vasopressin peptide in the samples can be calculated accordingly. The standard range in blood is 1 - 5 pg/mL, in intravascular vasopressin half-life, is about 15-26 minutes. Measurement of plasma ADH / VAS was done by the principle of “competitive” enzyme immunoassay method.

5.17. Reagents:

- 96 wells Vasopressin Microplate (12 strips x 8 wells) coated with secondary antibody (Item A)
- Concentrated wash buffer solution (Item B)
- Standard Vasopressin peptide (Item C)
- Anti-Vasopressin polyclonal antibody (Item N)
- Concentrated Assay diluent (Item E)
- Biotinylated Vasopressin peptide (Item F)
- Concentrated HRP-conjugated streptavidin (Item G)
- Positive control (Item M)
- Tetramethylbenzidine (TMB) one step substrate reagent (Item H)
- Stop Solution 0.2 M sulfuric acid (Item I)

5.18. Reagents preparations:

All the ELISA kit reagents were kept on ice during reagent preparation steps.

5.18.1. Preparation of Plate and Anti-Vasopressin Antibody:

1. Before opening the sealed microplate pouch equilibrate to room temperature
2. Label removable 8-well strips as appropriate for our experiment.
3. 5X Assay Diluent was diluted 5-fold with deionized or distilled water.
4. Briefly centrifuged the anti-Vasopressin antibody vial (Item N) and reconstituted with 50μl of 1X Assay Diluent to prepare the antibody concentrate and mixed correctly.
5. Anti-Vasopressin antibody working solution prepared by diluting the antibody concentrate to 100-fold with 1X Assay Diluent.

5.18.2. Preparation of Biotinylated Vasopressin:

The biotinylated Vasopressin vial was briefly centrifuged before use.

5.18.2.1. The preparation of working stock solution:

Entire contents from biotinylated Vasopressin ampoule was transferred to a tube which contains 10 ml of 1X assay diluent B and mixed thoroughly. The final concentration of biotinylated was 20 ng/ml.

5.18.2.2. Second dilution Biotinylated vasopressin for standards:

2ml of working stock solution was added to 2 ml of standard assay diluent. The final concentration of Biotinylated Vasopressin was ten pg/ml.

5.18.2.3 Second dilution Biotinylated Vasopressin for positive control:
100 μl of working stock solution was added to 100 μl of the prepared Positive Control. The final concentration of Biotinylated Vasopressin was ten pg/ml.

5.18.2.4. **Second dilution Biotinylated Vasopressin for test samples:**

125 μl of working stock solution was added to 125 μl of prepared sample. The final concentration of Biotinylated Vasopressin was 10 pg/ml.

![Diagram](image_url)

**Figure 17. Preparation of Biotinylated Vasopressin**

5.18.3. **Preparation of Standards:**

1. Labelled 6 micro tubes with the following concentrations: 1,000 ng/ml, 100 ng/ml, 10 ng/ml, 1 ng/ml, 100 pg/ml and 0 pg/ml. 450 μl of biotinylated Vasopressin working solution added into each tube, except the 1,000 pg/ml.

2. Briefly centrifuged the vial of Vasopressin standard. Reconstituted with ten μl of double distilled water and briefly vortexed. Eight μl of vasopressin standard and 792 μl of 10 ng/ml biotinylated vasopressin working solution was added to the tube labeled 1000 ng/ml. Mixed thoroughly. This solution works as the first standard (1000 ng/ml vasopressin standard, ten ng/ml biotinylated Vasopressin).
3. 100 pg/ml standard prepared by pipetting 50 μl of the 1000 ng/ml Vasopressin standard into the tube labeled 100 ng/ml. Mixed thoroughly.

4. Repeated above step with each successive concentration, the dilution series showed in the illustration below. Each time, 450 μl of biotinylated vasopressin and 50 μl of the prior concentration was used until the 100 pg/ml was reached. Each tube was mixed thoroughly before the next transfer.

![Figure 18. Plasma vasopressin standard preparations](image)

**5.18.4. Positive Control Preparation:**

Briefly centrifuged the positive control vial and reconstituted with 100 μl of double distilled water and 100 μl of working stock solution was added. The final concentration of biotinylated Vasopressin was ten ng/ml.

**5.18.5. Sample preparation:**

125 μl of test sample added to 125 μl working stock solution (Biotinylated Vasopressin). Total volume was 250 μl. It was enough for duplicate wells on the microplate. The final concentration of biotinylated Vasopressin was ten ng/ml.
5.18.6. Preparation of wash buffer:

The wash concentrate (20X) contains visible crystals, warmed to room temperature and mixed gently until it dissolved. Diluted 20 ml of wash buffer concentrate to yield 400 ml of 1X wash buffer by using deionized or distilled water. After using a partial plate diluted wash buffer stored at 2-8°C.

5.18.7. HRP-Streptavidin solution preparation:

The required amount of HRP-Streptavidin solution was prepared freshly just before use. HRP-Streptavidin concentrated vial spin and mixed gently before the use, as the precipitates may be formed during storage. Dilution of HRP-Streptavidin concentrate was done to 1000-fold with 1X assay diluent.

5.18.8. Assay procedure:

1. All the reagents kept on ice during the reagent preparation steps.
2. 100 μl of Anti-Vasopressin antibody added to each well. At room temperature, it was incubated for 1.5 hrs with gentle shaking (1-2 cycles).
3. Discarded the solution and washed for four times with washing buffer, 200 - 300 μl each well by using a multi-channel pipette. In each step, the solution is entirely removed to obtain an accurate result. After the last wash, wash buffer was completely removed by aspirating or decanting. Invert the microplate and blotted against the clean paper towel to ensure the complete dryness of wells.
4. 100 μl of each standard, positive control, and the sample was added to appropriate microplate wells, including blank. Covered wells and incubated for 2.5 hrs. at room temperature with gentle shaking (1-2 cycles).
5. Discarded the solution and washed for four times as explained above.
6. 100 μl of freshly prepared streptavidin solution is pipetted into each microplate wells and incubated for exact 45 minutes at room temperature with gentle shaking.

7. Discarded the solution and washing procedure followed as mentioned above.

8. 100 μl of TMB one-step substrate reagent added to each microplate well, and at room temperature in the dark, it was incubated for 30 minutes with gentle shaking (1-2 cycles)

9. In the end, 50 μl of stop solution added to each well and reading was taken at 450 nm in ELISA microplate reader immediately.

5.18.9. **Typical standard graph:**

The standard curve was constructed by plotting the absorbance OD (Y-Axis) of standards against the known concentration (X- axis) of standards ranging from 0.01, 0.1, 1, 10, 100, 1000 ng/ml in logarithmic scale, using the four parameter algorithm. Here the microplate reader performed the automatic calculations on analyte concentration. The result read as the total concentration of plasma renin in ng/ml.

![Figure 19. Standard graph of plasma Vasopressin (VAS)](image)
5.19. **Assessment of Quality of life by the EQ-5D-5L tool:**

EQ-5D-5L is a standardized measure of the status of health. It was developed by EuroQol group, which provides a simple generic measure of health for clinical and economic appraisal (274). It applies to a full range of health conditions and treatments; it offers a simple descriptive profile and also a single index value for health status which can be used in the clinical and economic evaluation of health care as well as in population health surveys (276).

Prior permission was taken from the EuroQol group to use Kannada and English version of EQ 5D 5L questioner. (EQ 5D- 5L questioner attached in annexure). EQ-5D-5L consists of 2 pages – First page EQ-5D-5L descriptive questioners and in second page EQ visual analog scale (EQ-VAS). It has five dimensions – Mobility, self-Care, usual activity, pain, and anxiety. However, each dimension has five levels: no problem, slight problems, moderate problems, severe problems and extreme problems. Visual analog scale was explained asked them to mark on the scale and noted the same value in the response sheet. The quality of life parameters was assessed in AIPE group, and ATNE group and same parameters were re-assessed in follow-up (CTG).