2. MATERIALS AND METHODS

2.1 Patients and Controls

The total number of participants for the entire study includes 170 subjects.

Phase I

Patient group: This group consists of 102 Rheumatoid arthritis patients (77 female : 25 male) attending the Rheumatology clinic at Sri Ramachandra Medical College & Research institute at Chennai fulfilling the 1987 ACR criteria for the diagnosis of rheumatoid arthritis.

Control group: 38 (28 female: 10 male) age matched controls from staff and faculties working in Sri Ramachandra University.

Phase II

Patient group: In this group 55(41 female; 14 male) out of 102 phase I RA patients were followed- up after one year.

Control group: 30 (23 female: 7 male) age matched controls.

The patients and controls were similar in ethnicity and nutritional habits. This study was approved by the Institutional Medical and Ethics committee (MEC/06/51/23)

Selection criteria for the study population

Inclusion criteria

Rheumatoid arthritis patients: subjects with age between 23-70 years. The primary inclusion criteria were definite RA fulfilling 1987 ACR criteria and disease duration of 6 months onwards.
Control group: subjects with age between 23-70 years who are free from articular, bone, liver or any other endocrine or chronic disease.

Exclusion criteria for both the groups

Subjects with habitual smoking, alcohol consumption, physiological status like pregnancy, lactation were excluded. Also subjects with known infectious disease, other diseases like diabetes mellitus, hypertension, thyroid dysfunction, neurological disorders, cancer and any other forms of arthritis were excluded from the study.

The patient group is further grouped with the help of rheumatologist as, Early RA- with the disease duration of less than two year from the onset of initial symptoms and late RA, with the disease duration of more than two year from the onset of initial symptoms.62

The patient history, physical activity and visual analogue score (VAS) were obtained by a personal interview of all the patients along with relevant clinical data and treatment history were collected using a health assessment questionnaire (HAQ). The number of swollen and tender joints (total joint count) was recorded using a goniometer with the help of rheumatologist. A copy of the HAQ is attached in the appendix.

Sample type: The blood samples of 5ml with and without anticoagulant was collected from rheumatoid arthritis patients and control after getting written informed consent (enclosed in annexure).

Serum sample collected was stored as aliquots in deepfreezer at (-80°C) at department of microbiology, Sri Ramachandra Medical College, Chennai, India.
Traditional markers of disease activity analyzed

Disease activity score (DAS28)
It was calculated using the universally accepted formula,

\[ \text{DAS 28} = 0.56 \sqrt{\text{(Number of tender joints)}} + 0.28 \sqrt{\text{(Number of swollen joints)}} + 0.7 \ln(\text{ESR: 1hour}) + 0.014 \times \text{VAS}. \]

Erythrocyte sedimentation rate (ESR)

ESR was estimated within an hour of collection by Westergren method (ICSH recommendations).

C-reactive protein (CRP)

CRP in serum is measured by using a latex agglutination test (Omega Diagnostics Ltd, Alloa, Scotland, UK). This is a semi-quantitative method with a detection limit of 6 mg/L.

The values for physical activity, visual analog score (VAS), number of swollen and tender joints (total joint count), disease duration, treatment details were recorded in the health assessment questionnaire (HAQ) under the guidance of rheumatologist.

Biochemical markers analyzed

- Anti-cyclic citrullinated peptides (Anti-CCP)
- Rheumatoid factor (RF)
- Hyaluronic acid (HA)
- YKL-40
- Matrix metalloproteinase (MMP-1)
- Cartilage oligomeric matrix protein (COMP)
- Osteocalcin (OC)
All these biochemical parameters were analyzed by \textit{in vitro} quantitative ELISA method in batches from the stored samples within 60 days.

**Statistical analysis**

Statistical analysis was carried out with SPSS version 15.0. Because most of the variables did not follow normal distribution all the statistical analysis was done using non parametric tests. The results of biochemical marker concentrations were expressed as median and range. Comparison of biochemical markers concentration between groups was calculated by non-parametric significant Kruskal-Wallis followed with Bon-ferroni adjusted Mann-whitney test for unpaired differences and Wilcoxon signed rank test was used to assess the changes in parameters studied at baseline levels and after one year. Correlations between different parameters were calculated by Spearman Rho test and \( p \) values of \( \leq 0.05 \) were considered significant.

2.2 METHODS FOR ESTIMATING BIOCHEMICAL MARKERS IN RHEUMATOID ARTHRITIS

2.2.1 Quantitative estimation of Anti-cyclic Citrullinated Peptides

Medizym Anti-CCP Medipan GMBH 15827 Berlin (Germany)-Medizym anti-CCP is used for the quantitative determination of IgG antibodies against cyclic citrullinated peptides (CCP) in human serum.

**Principle**

Anti-CCP is an enzyme immunoassay for the quantitative determination of IgG auto antibodies to cyclic citrullinated peptides (CCP).
in human serum. In the first step CCP auto-antibodies from the diluted sample (as well as from the calibrators and control) bind to cyclic citrullinated peptides coated on the microtiter plate. After an incubation of 60 minutes at room temperature (RT) unbound components are removed by washing. In a next step bound antibodies react with added anti-human IgG horseradish peroxidase (HRP) complex. Excessive conjugate is removed after 30 minutes at room temperature by another washing step.

HRP converts the colorless substrate TMB added into a blue product. The enzyme reaction is stopped by adding an acid solution after 15 minutes at RT. The color changes from blue to yellow. The absorbance of the resulting product is measured at 450/620nm within 30 minutes. The obtaining optical density (OD) is directly proportional to the amount of antibodies.

**Anti-CCP reagent preparation**

A) Allow samples to reach room temperature prior to assay. Patients samples have to be diluted 1 + 100. (e.g.: 5ul sample + 500ul sample diluent).

B) Prepare a sufficient amount of washing solution by diluting the concentrated wash buffer (B) 1 + 9 with distilled or de-ionized water. The diluted washing solution can be stored at 2-8°C up to 30 days.

**Assay procedure**

1. Pipette 100ul of calibrators and diluted patient sample and normal control serum into the corresponding wells.
2. Cover the plate and incubate for 60 min at room temperature (RT)
3. Aspirate or flick out by striking the wells sharply onto absorbent paper to remove any residual droplets.

4. Wash 3 times with 300ul washing solution with 5 seconds soaking time each.

5. Cover the plate and incubate for 30 min at RT.

6. Aspirate or flick out by striking the wells sharply onto absorbent paper to remove any residual droplets.

7. Wash 3 times with 300ul washing solution with 5 seconds soaking time each.

8. Add 100ul substrate solution to each well and shake shortly.

9. Incubate for 15 min in the dark at RT.

10. Add 100ul stop solution to each well.

11. Read the optical density (OD) at 450nm Vs 620nm within 30min after adding the stop solution.

**Data analysis**

The standard curve is established by plotting the mean OD values of the calibrators versus their respective Anti-CCP concentrations. The Anti-CCP concentrations of the controls and the diluted samples are directly read off in U/ml from the measured OD values at 450nm.

The analytical sensitivity was established to be 1.2U/ml.
2.2.2 Quantitative estimation of Rheumatoid factor (RF) IgG

RF IgG ELISA (Demeditec Diagnostics GMBH- D 24145 Kiel (Germany))

The DEMEDITAC Rheumatoid factor (RF) IgG ELISA test kit has been used for the detection and quantitative determination of RF in serum and plasma.

Principle

DEMEDITAC Rheumatoid factor (RF) IgG ELISA test kit is based on the principle of the enzyme immunoassay (EIA). Goat IgG is bound on the surface of the microtiter strips. Diluted patient serum, ready to use standards and controls are pipette into the wells of the microtiter plate. A binding between RF IgG of the serum and immobilized goat IgG takes place. After one hour incubation at room temperature, the plate is rinsed with diluted wash solution, in order to remove unbound material. Then ready-to-use anti-human IgG peroxidase conjugate is added and incubated for 30 minutes. After a further washing step the substrate (TMB) solute ion is pipetted and incubated of for 20 minutes, inducing the development of a blue dye in the wells. The color development is terminated by the addition of the stop solution, which changes the color from blue to yellow. The resulting dye is measured spectrophotometrically at the wavelength of 450nm. The concentration of RF IgG is directly proportional to the intensity of the color.
Reagent preparation

A) Preparation of sample buffer-Dilute the contents of each vial of the sample buffer concentrate (5X) with distilled water to a final volume of 100ml prior to use.

B) Preparation of wash solution-Dilute the contents of each vial of the buffered wash solution (5X) with distilled water to a final volume of 100ml prior to use.

C) Sample preparation-Dilute the samples 1:100 with sample buffer before assay (combine 10µl of sample with 990 µl of sample buffer in a polystyrene tube. Mix well. Controls are ready to use.

Assay procedure

Preparation of reagents: Washing solution: dilute before use 1+9 with distilled water. If during the cold storage crystals precipitate, the concentrate should be warmed up at 37°C for 15 minutes. All reagents and samples were brought to room temperature before use. Standards and samples should be assayed in duplicates. A standard curve should be established with each assay.

1. Prepare a sufficient amount of microtiter wells for the standards, controls and samples in duplicate as well as for a substrate blank.

2. Pipet 100µl each of the diluted (1:100) samples and ready to use standards and controls respectively into the wells. Leave one well empty for the substrate blank.

3. Cover plate with the re-usuable plate cover and incubate at room temperature for 60 minutes.
4. Empty the wells of the plate and add 300ul of diluted washing solution. This procedure is repeated totally three times. Rests of the washing buffer are afterwards removed by gentle tapping of the microtiter plate on a tissue cloth.

5. Pipet 100ul each of ready to use conjugate into the wells. Leave one well empty for the substrate blank.

6. Cover plate with the re-usable plate cover and incubate at room temperature for 30 minutes.

7. Empty the wells of the plate and add 300ul of diluted washing solution. This procedure is repeated totally three times. Rests of the washing buffer are afterwards removed by gentle tapping of the microtiter plate on a tissue cloth.

8. Pipet 100ul each of ready to use substrate into the wells. This time also the substrate blank is pipetted.

9. Cover plate with the re-usable plate cover and incubate at room temperature for 20 minutes.

10. To terminate the substrate reaction, pipet 100ul each of ready to use stop solution into the wells. The substrate blank is pipetted.

11. After thorough mixing and wiping the bottom of the plate, perform the reading of the absorption at 450nm and reference wavelength 620nm. The color is stable for at least 60 minutes.
Data analysis

The obtained OD of the standards is plotted against their concentration using 4 parameter logistics. The analytical sensitivity was established to be 0.67 IU/ml.

2.2.3 Quantitative estimation of Hyaluronic acid

Principle; The TECO assay kit for Hyaluronic acid TE1017 is a competitive assay using a HA- specific biotinylated binding protein (Biotin-HABP) and HA microtiterplates. This biotinylated binding protein binds to immobilized HA, if not saturated by free HA of the samples. In the following incubation step a streptavidin-peroxidase (SA-HRP) conjugate binds to the biotinylated binding protein. In the closing substrate reaction the colour development will be catalyzed quantitatively inversely dependent of HA levels of the samples.

The kit is provided with 6 standards, ready to use- low and high controls, sample diluent, reaction solution, Biotin-HABP, SA-HRP conjugate TMB substrate and stop solution. Wash buffer concentrate was prepared by diluting one vial of 30ml wash buffer concentrate by 1:30 with distilled water up to 900ml.

Reagent preparation

A) Allow the sealed micro titer plate to reach room temperature for at least 30 minutes.

B) 6 vials of standard native HA, 1vial of low control, 1vial of high control, 1vial of 10ml sample diluents all are ready to use.
C) 1vial of 10ml reaction solution, 1vial of 7ml biotinylated HA binding protein, 1vial of 12ml streptavidin peroxidase conjugate, 1vial of 12ml TMB substrate, 1vial of 12ml stop solution all are ready to use.

D) 1vial of 30 ml wash buffer concentrate (dilute the 1:30 concentrate with distilled water up to 900ml)

**Assay procedure**

1. Pipette 70µl assay buffer into each wells.
2. Add 30µl of each standards, controls and samples into the corresponding wells.
3. Add 50µl of Biotin-HABP and incubate the plate for 95min at room temperature.
4. After incubation aspirates the contents of the wells and wash 5 times with 350µl diluted buffer.
5. Pipette 100µl SA-HRP conjugate into each well and incubate the plate for 35min at room temperature.
6. After incubation aspirates the contents of the wells and wash 5 times with 350µl diluted buffer.
7. Pipette 100µl TMB-substrate solution into each well and incubate the plate for 30min in the dark at room temperature on a shaker (500rpm).
8. Stop the reaction by adding 100µl stop solution and measure the color reaction within 10 minutes at 450nm (reference interval 590-650nm).
Results were analyzed using 4 – parametric curve fit for automatic data reduction. The analytical sensitivity was 10.2ng/ml.

2.2.4 Quantitative estimation of YKL-40

Microvue YKL-40 EIA-QUIDEL corporation specialty products catalog no.8020

**Principle**- Microvue YKL-40 enzyme immune assay is a three step procedure; first the standards, controls and test specimens are added to micro assay plate coated with streptavidin along with the capture solution containing a biotinylated murine monoclonal antibody to human YKL-40. The monoclonal antibody binds to YKL-40 in the standards, controls and test specimens and the biotin binds to the avidin on the microwell plate, immobilizing the antibody. After the incubation period, wash to remove any unbound material. In the second step alkaline phosphatase (ALP) conjugated rabbit anti-YKL-40 is added to each well which will binds to the immobilized YKL-40 in the first step. Again after the incubation period, wash to remove any unbound material. In the third step p-nitro phenyl phosphate, a chromomeric substrate solution is added to assay well. The bound ALP reacts with the substrate forming a yellow color. After an incubation period the reaction is stopped chemically and color intensity is measured spectrophotometrically at 405nm. The intensity of the color is proportional to the concentration of YKL-40 present in the samples, controls and standards.

Results are calculated from the generated standard curve using linear regression analysis. Prepare the required amount of wash buffer by
diluting the 10X wash buffer ten fold with deionized water. And also reconstitute required vial of enzyme conjugate with 7ml of Reconstitute buffer. Prepare working substrate solution as per the assay procedure by vigorously shaking and allowing to dissolve for 30-60 minutes.

**Reagent preparation**

A) Preparation of wash buffer solution-Dilute the wash buffer solution (10X) in deionized water to prepare a 1X working solution.

B) Preparation of enzyme conjugate solution-Reconstitute each required vial of enzyme conjugate with 7ml of the Reconstitution buffer.

C) Preparation of working substrate solution-Prepare working substrate solution within one hour of use. Put one substrate tablet into each required bottle of room temperature substrate buffer as per the instruction manual. Allow 30-60 minutes for tablets to dissolve by vigorously shaking the bottles to completely mix.

**Assay procedure**

1. Pipette 20ul of standards, controls and specimens into assay wells within 40 minutes.

2. Pipette 100ul of capture solution into assay wells and incubate for 60min at 18-28°C.

3. Again pipette 100ul of enzyme conjugate and incubate for 60min at 18-28 °C.

4. Pipette 100ul of substrate solution and incubate for 60min at 18-28°C.
5. Add 250µl of working wash buffer to each well and manually empty the strips and repeat the procedure 3 times for a total of four washes.

6. Add 100 µl of reconstituted enzyme conjugate and incubate for 60min at 18-28°C.

7. Add 250µl of working wash buffer to each well and manually empty the strips and repeat the procedure 3 times for a total of four washes.

8. Add 100 µl of working substrate solution into each well and incubate for 60min at 18-28°C.

9. And finally pipette 100ul of stop solution and read the O.D at 405nm.

Analyze the assay results using the linear curve fit or using 4 parametric logistic software programmes. The standard curve for the YKL-40 EIA kit is also generated using each standard values (Y-axis) and assigned concentration for each YKL-40 (X-axis). The detection limit is 5.4ng /ml.

2.2.5 Quantitative estimation of Matrix metalloproteinases -1

**Principle:** The Ray Bio human MMP-1 ELISA kit is an *in vitro* enzyme-linked immunosorbent assay for the quantitative measurement of Human MMP-1 pro and active forms in serum, plasma. This assay employs an antibody specific for Human MMP-1 coated on a 96-well plate. Standards and samples are pipette into the wells by the immobilized antibody. The wells are washed and biotinylated anti-human MMP-1
antibody is added. After washing away the unbound biotinylated antibody, HRP-conjugated streptavidin is pipette to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of MMP-1 bound. The stop solution changes the color from blue to yellow and the intensity of the color is measured at 450nm.

Assay procedure

1. Bring all reagents and samples to room temperature (18-25°C). Assay diluent, diluted 5-fold with deionized or distilled water and used for dilution of serum.

2. Preparation of standard: briefly spin the standard vial and add 400µl 1X assay of assay diluent to prepare 0.1µg/ml standard. The powder is dissolved thoroughly by a gentle mix. Add 120 µl of standard into a tube with 546.7µl 1X assay diluent into each tube. Use the stock standard solution to produce a dilution series. Mix each tube thoroughly before the next transfer. Gently vortex to mix. 1X assay diluent serves as the zero standards.

3. Visible crystals of wash concentrate (20X) warm to room temperature and mix gently until dissolved. Dilute 20ml of wash buffer concentrate into deionized or distilled water to yield 400ml of 1X wash buffer.

4. Briefly spin the detection antibody vial before use and add 100µl of 1X assay diluent into the vial to prepare a detection antibody concentrate. Pipette up and down to mix gently. The detection
antibody concentrate should be diluted 80-fold with 1X assay diluent and can be used in assay procedure.

5. Add 100µl of each standard and sample into appropriate wells. Cover well and incubate for 150 minutes at room temperature. Discard the solution and wash 4 times with wash 1X wash solution. Wash by filling each well with wash buffer (330 µl) using auto washer. After the last wash, invert the plate and blot it against clean paper towels.

6. Add 100µl of 1X prepared biotinylated antibody to each well. Incubate for 1 hour at room temperature with gentle shaking. Discard the solution and repeat washing.

7. Add 100µl of 1X prepared Streptavidin solution into each well, Incubate for 45minutes at room temperature with gentle shaking. Discard the solution and repeat washing.

8. Add 100µl of TMB one step substrate reagent to each well, Incubate for 30 minutes at room temperature with gentle shaking. Add 50µl of stop solution to each well. Read at 450nm immediately.

9. Calculate the mean absorbance for each set of duplicate standards, controls and samples and plot the standard curve using sigma plot software. The sensitivity of MMP-1 is typically less than 8 pg/ml.
2.2.6 Quantitative estimation of cartilage oligomeric matrix protein

The RD194080200 Human cartilage oligomeric matrix protein (COMP) is a sandwich enzyme immunoassay for quantitative measurement of human COMP.

**Principle**

In the Biovendor Human cartilage oligomeric matrix protein ELISA standards, quality controls and diluted samples are incubated in micro plate wells pre-coated with monoclonal anti-human COMP anti-body. After 60 minutes incubation and washing, biotin-labeled second monoclonal anti-human COMP anti-body is added and incubated with captured COMP for 60 minutes. After another washing, streptavidin-HRP conjugate is added. After 30 minutes incubation and the last washing step, the remaining conjugate is allowed to react with the substrate solution (TMB). The reaction is stopped by addition of acidic solution and absorbance of the resulting yellow product is measured. The absorbance is proportional to the concentration of COMP. a standard curve is constructed by plotting absorbance values against concentration of standards and concentration of unknown samples are determined using this standard curve.

Human COMP master standard: Reconstitute the lyophilized master standard with dilution buffer just prior to the assay and let it dissolve at least 15 minutes with occasionally gentle shaking. The resulting concentration of the COMP in the stock solution is 128 ng/ml. Prepare set of standards (4 ng/ml-128 ng/ml) using dilution buffer. Dilute wash solution (10X) ten fold in distilled water to prepare a working solution.
Reagent preparation

A) Preparation of standard solution-Reconstitute the lyophilized master standard with dilution buffer just prior to the assay and dissolve it with occasional gentle shaking. Prepare set of standards using dilution buffer as per the manual.

B) Preparation of control solution-Reconstitute each quality control (high and low) with dilution buffer just prior to the assay; dissolve it with occasional gentle shaking. Reconstituted quality controls are ready to use without any dilution.

C) Preparation of wash solution (10X)-Dilute the wash solution (10X) in distilled water to prepare a 1X working solution (100ml wash solution + 900ml distilled water).

Assay a procedure

1. Pipet 100 µl of standards, quality controls, dilution buffer and samples in duplicates into the appropriate wells.

2. Incubate the plate at room temperature (25°C) for 1 hour shaking at 300 rpm on an orbital micro plate shaker.

3. Wash the wells three times with wash solution (0.35ml per well). After final wash, invert and tap the plate strongly against paper towel.

4. Incubate the plate at room temperature (25°C) for 1 hour shaking at 300 rpm on an orbital micro plate shaker.

5. Wash the wells three times with wash solution (0.35ml per well). After final wash, invert and tap the plate strongly against paper towel.

6. Add 100 µL of Streptavidin-HRP conjugate into each well.
7. Incubate the plate at room temperature (25°C) for 1 hour shaking at 300 rpm on an orbital micro plate shaker.

8. Wash the wells three times with wash solution (0.35ml per well). After final wash, invert and tap the plate strongly against paper towel.

9. Add 100 µl of Substrate solution into each well. Avoid exposing the micro titer plate into direct sunlight by covering the plate with aluminum foil.

10. Incubate the plate at room temperature (25°C) for 10 minute. The incubation time may be extended (up to 20 minutes) if the reaction temperature is below than 20°C. Do not shake the plate during incubation.

11. Stop the color development by adding 100 µl of stop solution.

Determine the absorbance of each well using a microplate reader set to 450nm preferably with the reference wavelength set to 630nm. The absorbance should be read within 5 minutes and the obtained OD of the standards are plotted against their concentration using 4 parameter logistics. Results are reported as concentration of COMP ng/ml in samples.

The analytical sensitivity was established to be 0.4ng /ml.

2.2.7 Quantitative estimation of osteocalcin

The Bio-Line human OST -ELISA is a solid phase enzyme amplified sensitivity immunoassay performed on breakable microtiterplates. The assay uses monoclonal antibodies directed against distinct epitopes of human osteocalcin. Calibrators and samples react with capture monoclonal
antibody (MAb 1) coated on microtiter well and with a monoclonal antibody (MAb 2) labeled with horseradish peroxidase (HRP).

After an incubation period allowing the formation of a sandwich coated (MAb 1- human osteocalcin- (MAb 2-HRP, the microtiterplate is washed to remove unbound enzyme labeled antibody. Bound enzyme labeled antibody is measured through a chromogenic solution, which is added and incubated. The reaction is read at the appropriate wavelength. The amount of substrate turnover is determined colorimetrically by measuring the absorbance which is appropriate to osteocalcin concentration. The calibration curve is plotted and OST concentration in samples is determined by interpolation from the calibration curve.

Reagent preparation

A. Calibrators: reconstitute the zero calibrator with 1.0ml distilled water and other calibrators with 0.5ml distilled water.
B. Controls: Reconstitute the controls with 0.5ml distilled water.
D. Working wash solution: prepare an adequate volume of working wash solution by adding 199 volumes of distilled water to 1 volume of wash solution. Use a magnetic stirrer to homogenize.
Assay procedure

1. Pipette 25μl of each calibrator, control and sample into appropriate wells.

2. Pipette 100μl of working anti-OST-HRP conjugate into all the wells.

3. Incubate for two hours at room temperature and after that aspirate the liquid from each well.

4. Wash the plate 3 times by dispensing 0.4ml of wash solution into each well and aspirating the contents of each well.

5. Pipette 100μl of the chromogenic solution into each well within 15 minutes following the washing step.

6. Incubate the microtiterplate for 30 minutes at room temperature.

7. Pipette 100μl of the stop solution into each well.

8. Read the absorbance at 450nm (reference filter 630nm or 650nm) within 1 hour and calculate the results using 4 parameter logistic function curve fitting software.

The detection limit was 0.08 ng/ml and the reference value of the assay procedure is 5-25 ng/ml.